The Role of PTPN2 in Intestinal Epithelial Cells in the Pathogenesis of Inflammatory Bowel Disease

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1 ABBREVIATIONS

AOM ..... azoxymethane

APCs ..... antigen presenting cells

BiP ..... binding immunoglobulin protein

CD ..... Crohn's disease

CRC ..... colorectal cancer

DCs ..... dendritic cells

DSS ..... dextran sodium sulfate

eIF2α ..... eukaryotic initiation factor 2

ER ..... endoplasmic reticulum

ERAD ..... ER-associated degradation

GI tract ..... gastro intestinal tract

GWAS ..... genome wide association studies

IBD ..... inflammatory bowel diseases

IECs ..... intestinal epithelial cells

IFNγ ..... interferon gamma

IL ..... interleukin

ILF ..... isolated lymphoid follicles

IRE1 ..... inositol-requiring enzyme 1

LPS ..... lipopolysaccharide

MDP ..... muramyl dipeptide

MHCII ..... major histocompatibility complex II

NOD2 ..... nucleotide-binding oligomerization domain 2

PAMPs ..... pathogen associated molecular patterns

PRRs ..... pattern recognition receptors

PTPN2 ..... protein tyrosine phosphatase non-receptor type 2

ROS ..... reactive oxygen species

SNP.....single nucleotide polymorphism

TER ..... transepithelial resistance

TJs .....tight junctions

TNF ..... tumor necrosis factor

UC..... ulcerative colitis

UPR..... unfolded protein response

XBP1.....X-box binding protein 1,

2 SUMMARY

In industrialized countries the incidence of Crohn's disease (CD) and ulcerative colitis (UC), the most common variants of inflammatory bowel diseases (IBD), is rising since the beginning of the 20th century. IBD is believed to be caused by an overreacting immune response towards otherwise commensal bacteria of the gut in genetical predisposed individuals, leading to chronic intestinal inflammation. Genome wide association studies identified more than 160 genetic risk factors that mediate IBD susceptibility indicating that IBD is a polygenetic disease. The gene encoding protein tyrosine phosphatase non-receptor type 2 (PTPN2) is one of these risk genes identified so far. PTPN2 is also involved in the predisposition to other autoimmune diseases such as rheumatoid arthritis and diabetes type 1.

Colon specimens of IBD patients show increased levels of PTPN2 protein. Furthermore, cell culture experiments proved that loss of PTPN2 in intestinal epithelia cells has detrimental effects on important cellular processes, that are known to be involved in IBD pathogenesis including autophagy, barrier function, and cytokine secretion.

One aim of this thesis was to examine the role of PTPN2 in the context of endoplasmic reticulum (ER) stress conditions. ER stress pathways are initiated upon accumulation of misfolded proteins in the ER lumen and malfunction of these pathways are important for the development of IBD. We used monocytes (THP-1) and colon epithelia cells (HT-29) which responded differently to siRNA mediated PTPN2 depletion. Whereas PTPN2 lacking monocytes became more

susceptible to tunicamycin induced ER stress, the absence of this phosphatase had beneficial effects on colon epithelia cells. This clearly showed the cell type specific role of PTPN2 under ER stress conditions.

Another objective of this thesis was to investigate the role of PTPN2 in intestinal epithelia cells *in vivo*. Therefore, we bred mice with a tissue specific knockout of PTPN2 in the intestinal epithelium (PTPN2xVilCre mice) and induced colitis by dextran sodium sulfate (DSS) treatment. In the setup of acute colitis, colonoscopy indicated more severe inflammation in PTPN2xVilCre animals compared to their wild type littermates. Moreover, endoscopic control after chronic colitis suggested a less severe inflammation in mice carrying the tissue specific knockout. These findings could not be confirmed by histological analysis of colon specimens. Nevertheless, aberrant crypt foci were observed more frequently in PTPN2 deficient mice, suggesting an increased susceptibility to colon cancer. In the azoxymethane (AOM)/DSS model nearly all animals developed aberrant crypt foci irrespective of genotypes. Hence, in colon epithelial cells PTPN2 plays only a minor role in inflammatory processes, suggesting that the organism is capable of compensating this shortcoming.

In summary, PTPN2 seems to be less crucial for intestinal epithelial cells compared to monocytes. Given that the highest PTPN2 expression is found in hematopoietic cells, this is not surprising. As shown by other studies, loss of PTPN2 in cells of the immune system results in severe phenotypes. Here, we demonstrate that its loss in intestinal epithelial cells can be compensated *in vivo* even during intestinal inflammation.

3 ZUSAMMENFASSUNG

Chronisch entzündliche Darmerkrankungen (CED) mit den beiden wichtigsten Varianten Morbus Crohn und Colitis ulcerosa treten seit Beginn des 20. Jahrhunderts vor allem in industrialisierten Ländern vermehrt auf. Bei diesen Erkrankungen handelt es sich um eine Überreaktion des Immunsystems gegenüber der normalen Darmflora in genetisch veranlagten Personen, welche zur chronischen Entzündung des Darms führen kann. Genomweite Assoziationsstudien haben bereits mehr als 160 genetische Risikofaktoren identifiziert, die zur Prädisposition beitragen. Unter diesen Risikogenen ist auch das Gen, welche die Protein Tyrosin Phosphatase Non-Rezeptor Typ 2 (PTPN2) kodiert. PTPN2 vermittelt auch eine Neigung zur Ausbildung anderer Immunkrankheiten wie rheumatoide Arthritis und Diabetes Typ 1 vermittelt.

In Darmbiopsien von CED Patienten findet man eine erhöhte Menge an PTPN2 Protein. Außerdem konnten Experimente in Zellkultur bereits belegen, dass sich in Darmepithelzellen ein Fehlen von PTPN2 negativ auf viele zelluläre Prozesse wie Autophagie, Barriere-Funktion und Zytokin-Ausschüttung auswirkt. Von all diesen Prozessen weiß man, dass sie bei CED eine wichtige Rolle spielen.

Ein Ziel dieser Arbeit war es, die Rolle von PTPN2 im Zusammenhang mit endoplasmatischem Retikulum (ER) Stress zu untersuchen. ER Stress Signalwege werden durch ein Anhäufung von falsch gefalteten Proteinen im Inneren des ER ausgelöst und Fehlfunktionen innerhalb dieser Signalwege sind für die CED Pathogenese von Bedeutung. Wir haben Experimente mit

Monozyten (THP-1) und Darmepithelzellen (HT-29) durchgeführt, die beide sehr unterschiedlich auf den siRNA vermittelten Verlust von PTPN2 reagierten. Während Monozyten mit PTPN2 Defizit anfälliger für Tunicamyin induzierten ER Stress wurden, hatte der Verlust positive Konsequenzen für Darmepithelzellen. Dies zeigt klar die Zelltyp spezifische Rolle dieser Phosphatase im Kontext von ER Stress.

Als weiteres Ziel sollte im Rahmen dieser Promotionsarbeit die Bedeutung von PTPN2 für Darmepithelzellen *in vivo* untersucht werden. Dazu wurden Mäuse gezüchtet, die im Darmepithel kein PTPN2 exprimieren (PTPN2xVilCre Mäuse) und diesen durch Verabreichung von Dextran Natriumsulfat (DSS) eine Kolitis induziert. Die Koloskopie nach einer akuten DSS Kolitis gab Hinweise auf eine gesteigerte Entzündung bei PTPN2xVilCre Tieren im Vergleich zu ihren wildtypischen Wurfgeschwistern. Die endoskopische Kontrolle nach der chronischen DSS Kolitis zeigte hingegen eine mildere Entzündung in den Tieren mit gewebespezifischem knockout. Histologischen Untersuchungen von Darmbiopsien konnten diese Unterschiede jedoch nicht bestätigen. Allerdings beobachteten wir vermehrt abnorme Krypten in PTPN2 defizienten Mäusen bei der chronischen DSS Kolitis. Dies ließ uns zunächst vermuten, dass in diesen Tiere ein erhöhtes Risiko für kolorektale Karzinome vorliegt. Die Kombination von Azoxymethan (AOM) Injektion und DSS Gabe konnte diese These jedoch nicht bestätigen, da nahezu alle Tiere unabhängig von ihrem Genotyp abnorme Krypten ausbildeten. In Darmepithelzellen spielt PTPN2 daher keine relevante Rolle für den Entzündungsprozess, der Mangel kann durch den Organismus offensichtlich kompensiert werden.

PTPN2 scheint also in Darmepithelzellen weniger wichtig zu sein als in Monozyten. Dies ist nicht sonderlich überraschend, wenn man bedenkt, dass die höchste PTPN2 Expression bekanntermaßen in blutbildenden Zellen zu finden ist. Andere Studien konnten bereits zeigen, dass ein Verlust der PTPN2 Expression in Immunzellen in starken Phänotypen resultiert. Wir zeigen hier, dass ein PTPN2 Defizit in Darmepithelzellen auch während einer Entzündung *in vivo* kompensiert werden kann.

4 INTRODUCTION

4.1 The human gastrointestinal tract

The human gastrointestinal tract (GI tract) is a tubular organ system reaching from mouth to anus. Its purpose is the digestion of food thereby absorbing nutrients to provide the body with energy. The indigestible remains are expelled as faeces. The whole human GI tract is about 9 m long. The transit time varies greatly and depends on the type of food and the amount of fluid intake and is individually different.

After mechanical crushing and moistening the food leaves the oral cavity and enters the stomach via oesophagus. In the stomach food is stored temporarily and mixed with mucus and gastric juice. The gastric glands produce about 2500 ml gastric juice every day, which contains digestion enzymes, hydrochloric acid, potassium chloride and sodium chloride. The low pH of 1.5 to 2 causes the death of most of the microbes in our food and provides the optimal conditions for pepsin, a digestive enzyme that degrades proteins. The stomach capacity varies between individuals, but is on average about 1.5 l. Peristaltic movement forwards the semi fluid mass to the duodenum[2].

The duodenum forms a C-shaped connection between the stomach and the small intestine (cf. **Figure 4-1**). Pancreas, liver and gallbladder assist in digestion by secreting enzymes, bile and hormones into the duodenum. Bile, which is produced in the liver and stored in the pancreas, functions as emulsifier thereby degrading lipids. The Brunner's glands of the duodenal mucosa release alkaline mucus containing a lot of bicarbonate to neutralize the acid pH of the chyme.

The rest of the small intestine is sectioned into jejunum and ileum but this distinction is artificial, having no clear anatomical border (cf. **Figure 4-1**). The upper 2/5 part is defined as jejunum and the lower 3/5 section is called ileum, while both parts together are about 5-7 m in length. Even though there is a smooth transition between jejunum and ileum, there are microscopic anatomical differences between the proximal start of the jejunum and the distal end of the ileum. In the duodenum and proximal jejunum the mucosa forms tall circular folds (plicae) and possess many villi standing close together. In the distal part of the ileum the plicae are more separated from each other and there is an increased occurrence of solitary lymph nodules within the mucosa.

The majority of fluid and nutrient absorption takes place in the small intestine[3]. Peristaltic movement of the smooth muscles within the small intestinal wall pushes the chyme into the large intestine.

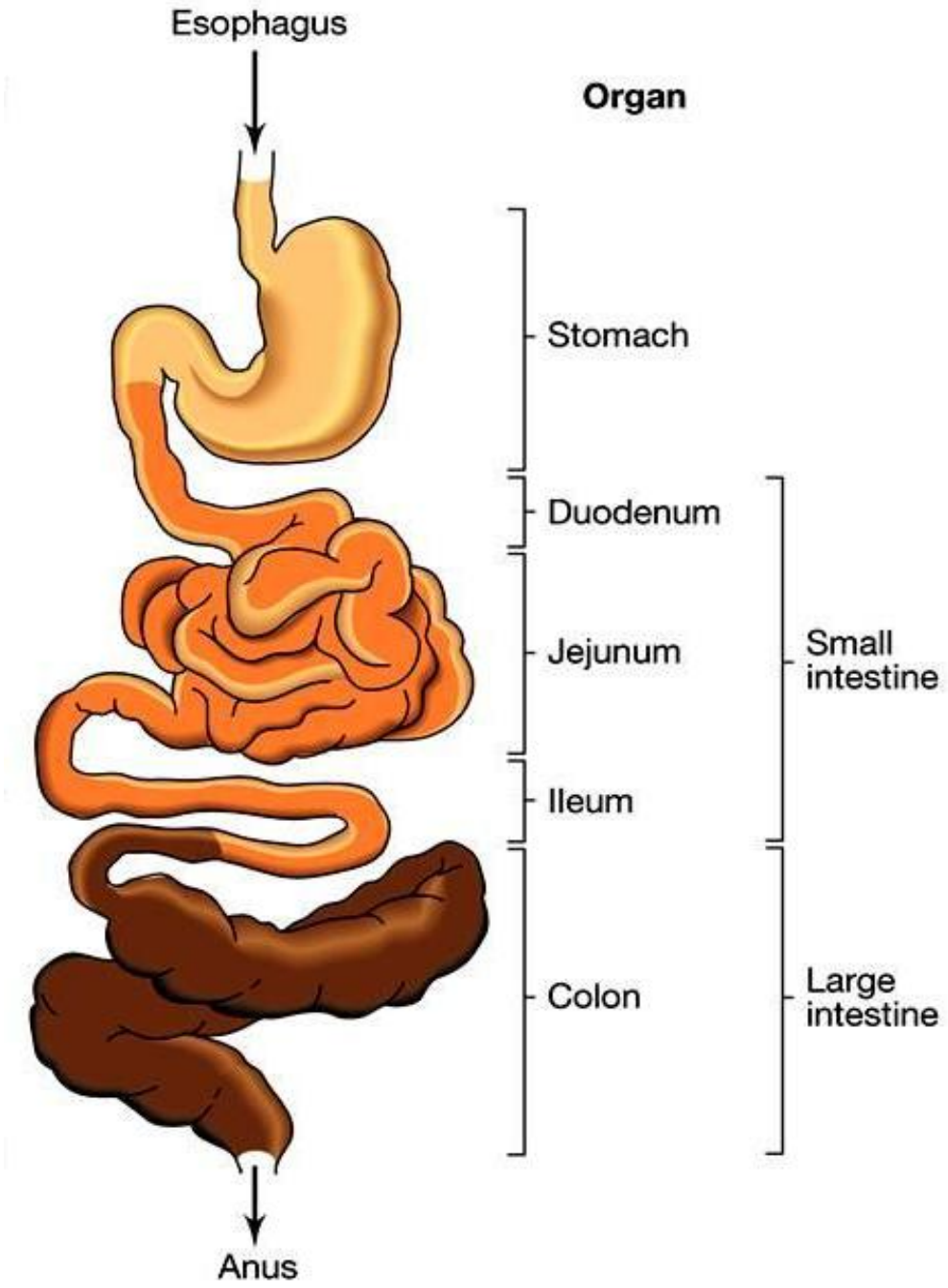

Figure 4-1 Structure of the gastrointestinal tract (image source: adapted from[1]). Ingested food enters the stomach via esophagus. The small intestine is subdivided into duodenum, jejunum, and ileum. From the large intestine only the colon is shown.

4.1.1 The colon – general function and microscopic architecture

The large intestine is divided into cecum, colon, rectum and anal canal. The cecum is a dead-end pouch between ileum and colon. The cecum of herbivores is enlarged and contains many bacteria that aid in digestion of cellulose.

In the colon, complex carbohydrates and also some proteins can be fermented by the huge population of bacteria that reside here[4]. Humans live in a symbiotic relationship with the myriads of bacteria that form our gut microbiota and produce short-chain fatty acids from otherwise indigestive food residues. The salvaged nutrients are then absorbed by intestinal enterocytes, the cell type that is also responsible for water homeostasis. The colon is substantial for water absorption, thereby producing about 200 g solid stool per day[5]. In contrast to the small intestine, the colon mucosa does not form villi. Peristaltic movements, created by the circular muscles in the colon wall, force the faeces through the colon to the rectum. Here it is stored before defecation takes place through the anus.

The colon wall is built of three layers: the muscularis externa, submucosa and mucosa (cf. **Figure 4-2**). As the most outer layer, the **muscularis externa** is embedded in loose connective tissue that holds the colon in its place within the peritoneal cavity. The muscularis externa consists of an inner circular and an outer longitudinal smooth muscle layer and is important for peristaltic movement. The **submucosa** is rich in blood and lymphatic vessels, nerve fibers and collagen fibrils that supply the mucosa. The mucosa represents the most inner layer and contains all the different cell types that are important for colon function and homeostasis.

The **mucosa** is further subdivided into three layers: the epithelium, lamina propria and muscularis mucosae (cf. **Figure 4-2**).

A single layer of epithelial cells separates our gut tissue (and subsequently our body) from the environment. The **epithelium** forms a physical and chemical barrier and can interact with commensals and pathogens (cf. chapter 4.1.3). The inner surface of the colon is enlarged by gland-like infoldings, the crypts of Lieberkühn. These crypts are covered by epithelium, which contains multiple cell types: enterocytes (water and electrolyte absorption), goblet cells (mucus secretion), enteroendocrine cells (hormone secretion), and stem cells.

The pluripotent intestinal **stem cells** reside at the crypt base and give rise to all epithelial cell types. The colon epithelium is highly proliferating, leading to a complete turnover every 24-96 hours. The stem cells divide in the basal crypt areas and migrate up to the tip of the crypt thereby differentiating and maturing.

The **enterocytes** represent the most abundant cell type of the epithelial lining. This absorptive cell type has a columnar shape and possesses microvilli on its apical surface to further increase surface area. Its main function is the absorption of water, ions, sugars, peptides, lipids and vitamin B12.

Goblet cells are needed for secretion of mucus and antimicrobial peptides. Mucus covers the epithelium and represents the very first line of defense against invaders. Its most important component is MUC2, a large protein that is highly glycosylated and forms a gel like polymer (together with other glyco-proteins). Antimicrobial peptides such as defensins get stuck in the mucin network, which leads to high antimicrobial activity at the epithelial surface. Therefore, the mucus

layer is important for regulating the mass and diversity of living bacteria that occupy our gut.

Enteroendocrine cells can release hormones and peptides that can act locally or systemically. They also function as chemoreceptors and can detect noxious compounds. Hence, this cell type plays a role in regulating digestion and connects the gut with the central nervous system.

Underneath the epithelium the **lamina propria** is located, a layer of loose connective tissue. There is a great number of different cell types within this connective tissue, for example: fibroblasts, lymphocytes, plasma cells, macrophages, and mast cells. The number and composition of cell types in the lamina propria depends on the physiological or pathological state. However, the majority of inhabitant cell types are tissue resident macrophages and IgA secreting B-cells. The abundance of immune cells makes the lamina propria a key spot for immune defense against invading microbes.

The **muscularis mucosae** separates the mucosa from the underlying submucosa. It is composed of several thin layers of smooth muscle fibers oriented in different ways. It guarantees the fine tuning of the intestinal surface and holds the mucosal glands in a constant state of soft tension to support the secretory activity. It is also needed to enhance contact between the epithelium and the luminal content.

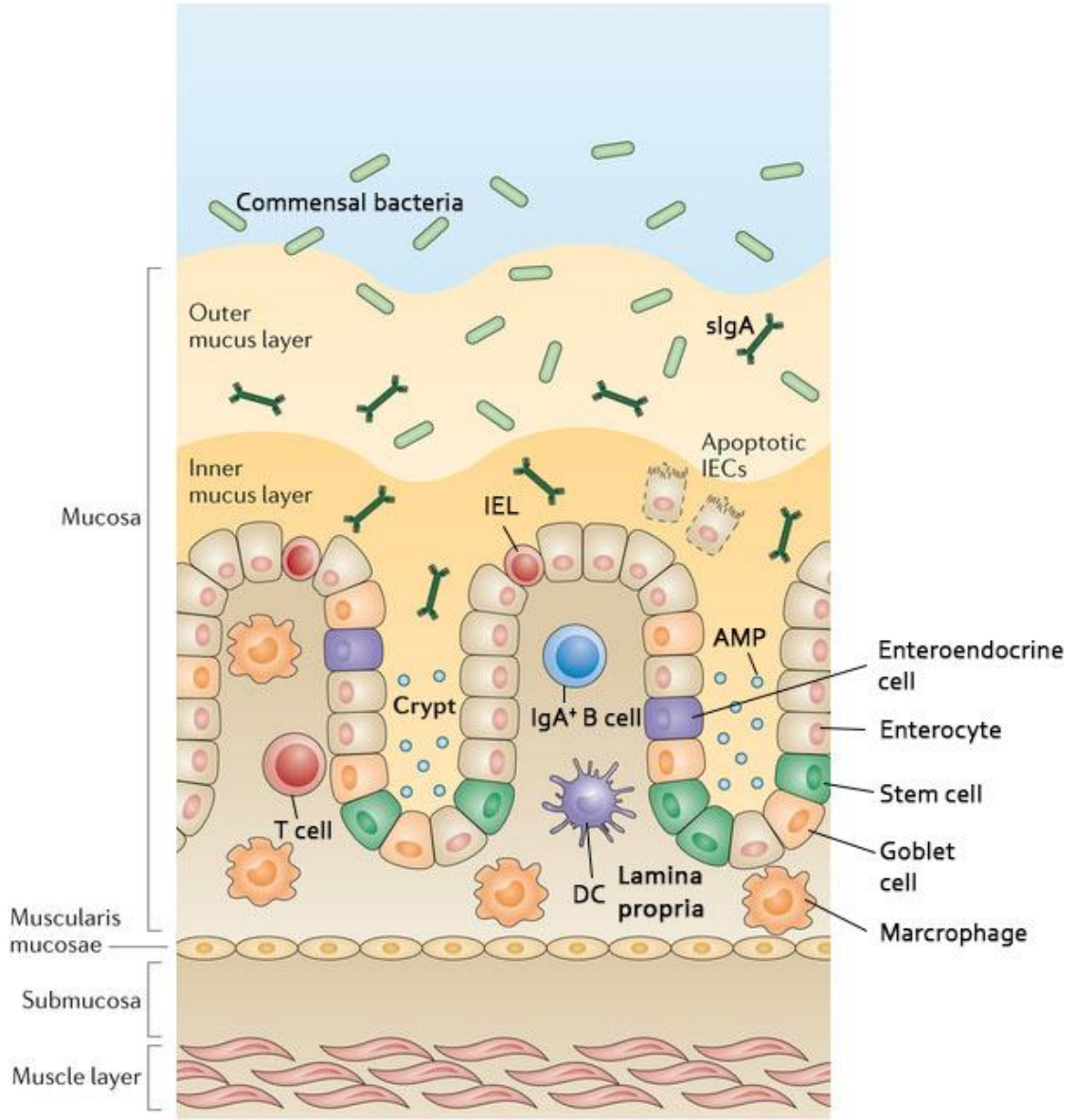

Figure 4-2 Microscopic anatomy of the colon wall (image source: adapted from[6]). The colon wall is built of 3 layers: an external muscle layer, the submucosa and the mucosa. The mucosa contains all the different cell types that mediate colon and immune function. The luminal surface is covered by a mucus layer, secreted by goblet cells. The epithelial lining is formed by enterocytes (water absorption), goblet cells (mucus secretion) enteroendocrine cells (hormone secretion) and stem cells (renewal of the epithelium).

4.1.2 The immune system of the colon

The intestine is the place with the largest contact area between body and external environment[7]. The heavy duty of the intestinal immune system is the discrimination between commensals, food and body's own tissue on the one hand and pathogens on the other hand. Disturbances in this homeostasis result in inflammation[8]. The mesenteric lymph nodes are the secondary lymphoid structures of the colon. The bean-shaped lymph nodes consist of a cortex with separated areas for B- and T-cells and a medulla where a lot of antibodies producing plasma cells reside. Antigen presenting cells migrate to the lymph nodes to activate lymphocytes.

There are also isolated lymphoid follicles (ILF), tertiary lymphoid structures, in the colon. They develop after birth in response to commensal microbes and contain mainly B-cells. During inflammation the lymph nodes and ILF enlarge due to hyperproliferating lymphocytes that undergo clonal expansion. There are also lymphocytes within the epithelial layer. These cells are important for reconstitution after tissue damage [9, 10] and they can stimulate AMP secretion from IECs[11]. The main immune effectors are the immune cells scattered in the LP: macrophages, lymphocytes, DCs and mast cells.

Macrophages are cells of the innate immune system, which help to eliminate invading microbes by phagocytosis and can initiate an immune response by secreting cytokines. They possess pattern recognition receptors (PRRs) that recognize pathogen associated molecular patterns (PAMPs) on microbes such as lipopolysaccharide (LPS) or muramyl dipeptide (MDP). Regarding lymphocytes, in the lamina propria there are mainly memory T-cells and IgA

producing plasma cells. The mucosa is literally drenched in IgA, which is important for homeostasis. IgA helps in eliminating pathogens without inflammation and is actively translocated to the luminal surface by IECs. Memory T cells are easier and faster to activate then naïve T-cells which immediately start to secrete effector cytokines such as interferon γ (IFNγ), IL-10 or TGFβ after recognizing their specific antigen. Dendritic cells (DCs) sample antigens from their environment by macropinocytosis. When activated by pathogens they stop macropinocytosis and migrate to the lymph nodes. There, they present the sampled antigens to lymphocytes. As all antigen presenting cells (APCs), DCs are able to detect pathogens via PRRs. Mast cells are huge and filled with toxic granulae which can be quickly released when the cells become activated.

Intestinal epithelial cells (IECs) also perform immune functions by secreting pro-inflammatory cytokines to recruit immune cells. Furthermore, IECs express major histocompatibility complex II (MHCII) molecules but do not express costimulatory signals under physiological conditions[12]. MHCII is classically expressed by APCs to present extracellular antigens to lymphocytes. Without the costimulatory signal the lymphocytes become anergic. This way tolerance is induced and immune activation is suppressed[13]. This mechanism is called oral tolerance and is important to suppress immune responses against commensals or food antigens. Just like APCs IECs also express PRRs, those enable them to recognize invading microbes.

In most cases, the innate immune system is capable to fight the invader. This is mainly done by macrophages that sense bacteria and other invaders via PRRs followed by phagocytosis. If this doesn’t result in victory over the pathogen,

macrophages and mast cells secrete cytokines like tumor necrosis factor (TNF), interleukin (IL-) 6 and IL-1 which initiate an inflammation. In the same time DCs activate the adaptive immune response by presenting antigens to lymphocytes in the mesenteric lymph nodes. The lymphocytes then start clonal expansion and several days after infection the cells are ready to fight. An important feature of inflammation is the recruitment of neutrophils to the side of infection. They fight the invaders by phagocytosis and by secretion of reactive oxygen species (ROS). They are chemo attracted to the side of inflammation by IL-8, another pro-inflammatory cytokine secreted by macrophages and many other cell types. As the most potent macrophage activator, IFNγ has also to be mentioned. Activated macrophages are more efficient in phagocytosis.

4.1.3 Barrier function

The mucosa separates the body from the environment, thereby forming a barrier towards invading microbes. This barrier is made of three components: the mucus layer, the epithelial cell layer and the tissue resident macrophages within the lamina propria.

The mucus is made of two layers. Whereas the outer layer is gel like and contains few bacteria, the inner layer is more viscous and nearly sterile. Antimicrobial peptides secreted by IECs and goblet cells are trapped in the inner layer, ready to defeat bacteria that are able to overcome the outer coat. IECs also secrete HCO3- and trefoil factor 3. Therefore the mucus is rich in bicarbonate to maintain a neutral pH at the epithelial surface. TFF3 crosslinks mucins and promotes epithelial repair[14].

Invaders that are able to pass through the mucus, face the firm layer of epithelial cells. To pass this barrier on a paracellular way, bacteria have to squeeze through gap between cells. Neighboring IECs are connected to each other via desomosomes, adherens junctions and tight junctions. Tight junctions (TJs) form the firmest contact, thereby determining the strength of this barrier. Furthermore, TJ separate the apical from the basolateral membrane which differ in protein and lipid composition and physiological function. This way TJ assist in creating cell polarity. TJ form a network of punctuate membranes fusions of neighboring cells, so called fibrils. There are transmembrane proteins such as occluding, claudins and JAM proteins whose extracellular domains are linked to the ones of neighboring cells. These TJ proteins are linked to an aggregation of intracellular membrane proteins, zonula occludens proteins, some of which are linked to the cytoskeleton.

TJ are highly regulated structures. There is a direct correlation between fibril numbers and barrier integrity. The TJ ”kisses” are impermeable structures with channels or pores which can open and close. TJ can be modified by number of kisses, channel openings and physical pore changes and can be regulated for example by meal related nutrients. TJ disruption contributes to diarrhea by leak flux. During transepithelial migration of neutrophils, TJ have to be opened and resealed afterwards. Furthermore, TJ are influenced by cytokines. IFNγ leads to internalization of TJ proteins thereby reducing barrier function.

Bacteria that passed the mucus and the IEC layer arrive in the lamina propria where they are phagocyted by macrophages. Monocytes circulate in the blood and eventually enter the colon or other tissue where they differentiate into tissue

resident macrophages. Once matured, they reside in the lamina propria of the colon.

4.2 Inflammatory Bowel Diseases

Inflammatory bowel diseases (IBD) are a group of chronic and relapsing inflammatory conditions of the small and/or large intestine with the two main variants Crohn's disease (CD) and ulcerative colitis (UC). The manifestations of IBD depend on the area of intestinal tract involved and the symptoms are not specific. Typically, there are symptom free periods between relapses. Patients often suffer from diarrhoea with urgencies during night and incontinence with mucus or blood containing stool. They further have abdominal pain and cramping, nausea and vomiting. These symptoms often lead to weight loss.

Despite having many things in common, there are some differences between CD and UC. CD can affect any part of the gastrointestinal tract but usually the distal ileum and colon are involved. It occurs as "skip lesions" between healthy areas (cf. **Figure 4-3**). Cobble stone pattern can typically be observed in colonoscopy and the inflammation affects all layers of the gut wall. UC is marked by a continuous inflammation starting from the rectum but is restricted to the colon. Usually, only the mucosa is inflamed whereas the other layers of the gut wall are not affected[15]. Colonoscopy is the most important test for diagnosing IBD. Even small ulcers and mild inflammation can be seen this way and biopsies for histological analysis can be taken.

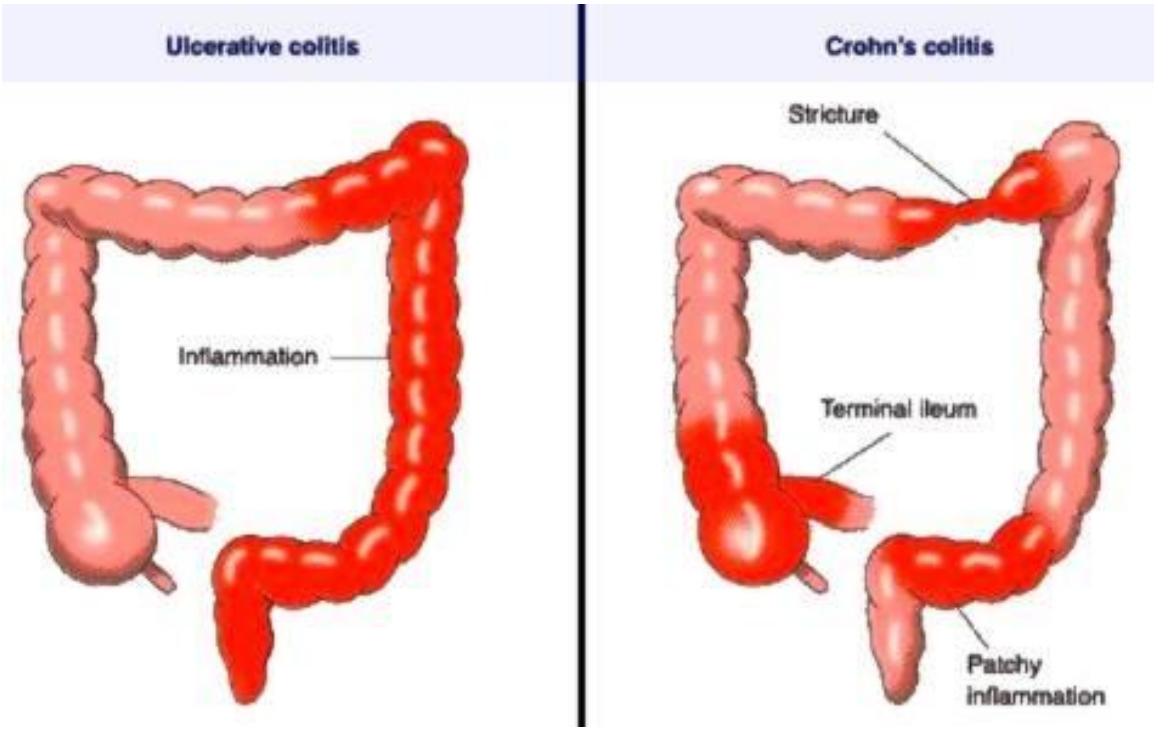

Figure 4-3 Distribution of inflammation in ulcerative colitis and Crohn's disease (image source:[16]). In ulcerative colitis the inflammation spreads from the rectum and is restricted to the colon, thereby only affecting the mucosa (left panel). Crohn's disease is characterized by inflammatory patches that can affect any part of the gastrointestinal tract. Furthermore, the inflammation affects the whole intestinal wall.

When IBD causes severe damage to the colon, it may be needed to be surgically removed. This can cure UC but not CD, because in CD the inflammation can reoccur in other parts of the gastrointestinal tract. A complication that often occurs in CD is the formation of fistulae. Deep ulcers can form abscesses that can break through to an adjacent organ creating a fistula.

People with IBD may have extra intestinal manifestations such as mouth sores and skin problems, arthritis, eye problems that affect vision. Furthermore, longstanding inflammation is a risk factor for the development of cancer. When stressed, lamina propria macrophages release pro-inflammatory signals that

may lead to increased probability of developing cancer. An example of this is the over activation of the IL6/STAT3 pathway which has been linked to colitis-associated cancer[17]. Colorectal cancer (CRC) is the third most frequent cancer and the third leading cause of cancer death in the United States[18].

Cytokines play a pivotal role in the development of IBD. The pro-inflammatory cytokine TNF is a hallmark in IBD. It is found to be increased in the mucosa of IBD patients and correlates with clinical and laboratory indices[19]. Anti-TNF antibody treatment is an established therapy in steroid-refractory CD patients, in fistulising CD and also in active UC[20]. Introduction of anti-TNF therapies in 1998 had a major impact on IBD management and prognosis[21]. But the side effects are fatal, due to its immune suppressive effects TNF blockers bear the risk for severe infections.

IL-6, another central cytokine in immunity, is found to correlate with clinical and histological severity of CD and UC[22, 23]. After steroid treatment mediated remission, elevated IL-6 serum levels are highly predictive for a relapse of the disease[24, 25]. Therefore, IL-6 is an important biomarker in IBD patients. The main sources of IL-6 are macrophages and $CD4^+$ T-cells. The majority of TNF/IL-1/IL-6 induced pathways are mediated by NFκB and MAPK.

Another cytokine that also crucial in chronic inflammation is IL-1β, which can be detected in the mucosa of IBD patients with active disease and correlates with inflammation severity[26-28]. The main source of IL-1β in colonic mucosa of IBD patients are stimulated macrophages.

Furthermore, IFNγ should be mentioned as an important cytokine in IBD. As noted in chapter 4.1.3, IFNγ stimulation results in barrier defects by disrupting TJ[29]. It also an important activator of macrophages, inhibits IEC migration[30] and impairs wound healing[31, 32].

4.2.1 The multifactorial pathogenesis of inflammatory bowel diseases

IBD was seldom seen before the rise of improved hygiene and urbanization at the beginning of the 20th century. There is a rising incidence and prevalence especially in industrialized nations[33]. The onset of IBD typically occurs at 15 to 30 years of age but can occur at any time in life. There is a bimodal age distribution with a second smaller peak at the ages of 50 – 70 years[34]. Until now there is no cure to IBD and normally it requires a lifetime of care.

Although, the multifactorial pathogenesis is still not entirely understood, it is generally accepted that IBD results from an uncontrolled inflammatory immune response towards otherwise commensal microbiota in genetically predisposed individuals. Genetic factors only account for 20-25 % of the heritability found in population based studies, leaving much room for environmental factors to contribute. This is supported by the fact that IBD is more common in Westernized countries than in non-industrialized countries. As individuals move from areas of low to high prevalence of IBD, fist-generation offspring acquire the same risk as the local population[35]. Therefore, IBD seems to be a result of the "westernized" lifestyle that is marked by changes in diet, smoking behaviour, variances in exposure to sunlight, pollution and industrial chemicals[36]. Hence, IBD is a disease of cleanliness, it demonstrates an inverse relationship with the degree of sanitation: poor sanitation appears to protect against IBD. Improved

hygiene alters the intestinal flora by decreasing exposure to certain critical bacteria. Smoking is the strongest environmental risk factor for Crohn's disease but is protective in ulcerative colitis: current smoking protects against UC (40% reduced risk to develop UC), whereas ex-smoker show higher risk to develop UC (1,7 times increased) than those who never smoked[37].

A topic that recently aroused much interest and that may link genetic and environmental factors is the role of the microbiota[38]. The microbiota of our gut is influenced by nearly everything in our environment, by everything we eat or drink. The microbiota itself can profoundly alter our immune composition and IBD patients show different microbiota compared to healthy individuals[38]. Also, in the mouse models of IBD the microbiota is crucial for disease development. Mice kept under germ free conditions don´t show inflammation in the model of chemical or genetically induced colitis.

4.2.2 Risk Genes

Epidemiological and family studies demonstrated the involvement of genetic factors, but nevertheless IBD cannot be explained by a single gene model alone[39]. It is not surprising that UC and CD share some but not all susceptibility loci. The disease phenotype is determined by several factors including interaction between allelic variants at a number of loci as well as genetic and environmental influences[39]. The presence of one mutated gene is not sufficient to predict IBD[40].

Approximately 30% of IBD-related genetic loci are shared by UC and CD, indicating common pathways[41]. These are normally pathways that are crucial for

intestinal homeostasis such as barrier function, epithelial restitution, microbial defence, innate immune regulation, ROS generation, autophagy, regulation of adaptive immunity, endoplasmic reticulum (ER) stress and metabolic pathways associated with cellular homeostasis[41]. More than 50% of IBD susceptibility loci have also been associated with other inflammatory and autoimmune diseases[41].

The strongest association with IBD was found for a single nucleotide polymorphism (SNP) within gene encoding the nucleotide-binding oligomerisation domain 2 (NOD2), an intracellular pattern recognition receptor that is activated by MDP, a peptidoglycan common to all bacteria. If NOD2, expressed by leukocytes and IECs, is not properly functioning, more bacteria are able to invade the tissue and can provoke inflammatory immune response.

X-box binding protein 1 (XBP1) is an example for a susceptibility gene of the ER stress pathway. This transcription factor is activated via the ER stress sensor inositol-requiring enzyme 1 (IRE1) by differential splicing (cf. chapter 0). Dysfunctional XBP1 in IECs leads to unresolved ER stress, which renders the cells more prone to inflammation[42].

This thesis is focussed on the IBD risk gene encoding protein tyrosine phosphatase non-receptor type 2 (PTPN2) that was also found to be associated with diabetes type 1 and rheumatoid arthritis.

4.3 Protein Tyrosine Phosphatase Non-Receptor Type 2

The protein tyrosine phosphatase non-receptor type 2 (PTPN2) is a phosphatase that removes phosphate groups from proteins at their tyrosine residues. Phosphatases are important enzymes in signal transduction. If the cell receives a signal from the environment due to ligand binding at a surface receptor, this leads to changes in conformation of the intracellular part of the receptor. This changed conformation enables the receptor protein to act as a kinase and to phosphorylate a target protein, thereby activating it. To end the signal, phosphatases are needed to remove the phosphate groups to set the enzymes back into an inactive state.

The phosphatase PTPN2 is ubiquitously expressed in all embryonic and adult mammalian tissues, but can be found in particular high amounts in cells of hematopoietic origin[43]. The PTPN2 gene is located on chromosome 18 in humans and in mice within a syntenic region[44]. In humans there are two common isoforms generated by alternative splicing: the 45 kDa variant is located in the nucleus whereas the 48 kDa form is found in the cytoplasm bound to the ER[45]. The 48 kDa isoform was first cloned from a human T-cell cDNA library[43] and therefore PTPN2 is sometimes also called T cell protein tyrosine phosphatase (TCPTP). For catalytic activity the nuclear variant seems to be more important. Upon activation it can exit the nucleus and attain to the cytoplasm where it can dephosphorylate target proteins. It has many known targets, the most important ones are: epidermal growth factor receptor (EGFR), insulin receptor and the signal transduction and activators of transcription molecules STAT1, 3 and 5.

The PTPN2 knockout mouse was the first mouse carrying the knockout of a protein tyrosine phosphatase generated nearly 20 years ago[46]. The recent development of conditional knockout mouse models[47-49] helped to identify a great number of phenotypes related to PTPN2 depletion. PTPN2 has been found to act on a broad list of physiological events such as inflammatory response[50], hematopoietic stem cells renewal[51], insulin signaling[52] and leptin regulation[53].

4.3.1 PTPN2 in IBD

Besides the correlations that were found in genome wide association studies (GWAS), there are some experimental findings that connect PTPN2 to IBD. First, PTPN2 protein expression is increased in colon tissue specimens of patients that suffer from active CD[54, 55]. This finding is strengthened by experiments on IEC cell lines that show an elevated PTPN2 expression after stimulation with the IBD associated pro-inflammatory cytokines IFNγ and TNF[54, 55]. Furthermore, PTPN2 deficiency induced by siRNA in IECs leads to an aggravated increase in IFNγ induced barrier defects[54]. Those barrier defects manifests in increased transepithelial resistance (TER), increased Claudin2 (a pore forming TJ protein) expression and increased permeability to FITC-dextran[54]. TNF stimulation of PTPN2 deficient IECs leads to exaggerated increase in secretion of the pro-inflammatory cytokines IL-6 and IL-8.

Epidermal growth factor (EGF) stimulation of IECs that are transfected with PTPN2 siRNA show increased EGFR phosphorylation[56]. This points to the fact that PTPN2 boosts EGF induced suppression of Ca2+ dependent Cl- secretion[56]. Carrying a PTPN2 mutation could therefore contribute to diarrhoea in IBD patients.

PTPN2 is also involved in the regulation of autophagy, a cellular process responsible for degradation of cellular components. *In vitro* studies demonstrate that IECs with knocked down PTPN2 expression are not capable of functional autophagy[57]. Upon infection with *Listeria monocytogenas*, this impaired autophagy results in ineffective clearance of bacteria[57]. Primary colonic lamina propria fibroblasts isolated from CD patients that carry the IBD associated PTPN2 variant behave the same way[57]. Furthermore, biopsies from active inflamed regions feature aberrant LC3BII expression patterns, indicative for dysfunctional autophagy[57].

Taken together this shows the importance of PTPN2 for maintaining the intestinal barrier.

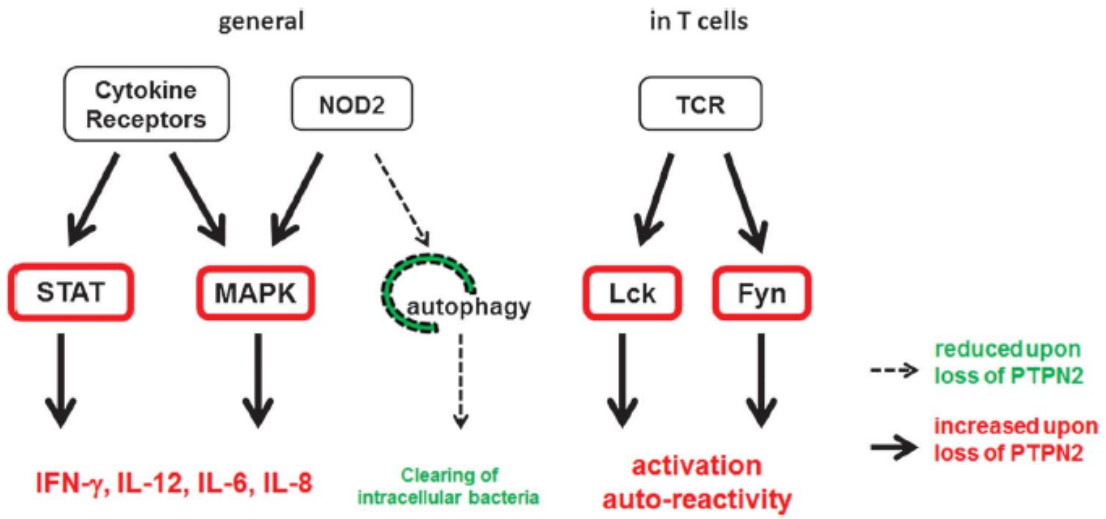

Figure 4-4 Signaling pathways regulated by PTPN2 (image source: adapted from[58]). PTPN2 deficiency leads to increased cytokine secretion by dephosphorylation of STAT and MAPK molecules. In addition, PTPN2 is essential for autophagy. Lack of PTPN2 in T cells results in exaggerated activation of TCR-associated kinases resulting in loss of tolerance towards self-antigen.

In THP-1 cells PTPN2 expression is elevated by IFNγ stimulation accompanied by decreased phosphorylation of STAT1 and STAT3[59]. In PTPN2 deficient circumstances IFNγ treatment causes increased levels of phosphorylated STAT1 and STAT3 and MAPK activity as well as increased secretion of the macrophage attractant cytokines IL-6 and MCP1[59]. The importance of PTPN2 in monocytes is also emphasized by other experiments. PTPN2 knockout mice die from systemic inflammation and exhibit high serum levels of IFNγ and intense tissue infiltration by monocytes[50]. Isolated macrophages of these animals are hypersensitive to LPS[50].

PTPN2 was discovered by screening a human T cell cDNA library and it is known to be expressed at high levels in T cells. Therefore it is not surprising that PTPN2 plays a major role in this cell type. Mice with a T cell specific PTPN2 knockout display strong inflammation and autoimmunity which can be passed on wild type mice by transfer of $CD8^+$ cell[59]. The mice with T cell specific knockout possess TCRs with lowered threshold for CD8+ proliferation[49]. In PTPN2 competent naive T cells, PTPN2 expression restricts homeostatic proliferation[60]. When CD8+ cells with PTPN2 deficiency are transferred into wild type recipients the T cells acquire features of antigen experienced cells[60]. The elevated TCR response contributes to a distorted TCR repertoire that results in autoimmunity[60]. Furthermore, when OVA specific T cells are transfected into mice that express OVA in β-cells this causes islet destruction in the pancreas only when co-transfected with OVA specific $CD4^+$ cells[61]. If PTPN2 deficient OVA specific CD8+ cells are transfected, this also gives rise to β-cell destruction and

diabetes[61]. This reveals that PTPN2 is involved in mediating peripheral T cell tolerance.

The PTPN2 knockout mouse clarifies the relevance of this phosphatase for haematopoiesis and immune function. Mice heterozygous for PTPN2 have bone marrow defects and impaired B and T cell function but the development of myeloid cells and macrophages is normal[46]. Bone marrow transfer from wild type donors to heterozygous PTPN2 mice does not rescue the phenotype, thereby illustrating possibility that hematopoietic defects are probably caused by stromal abnormalities[46]. Another study could show that PTPN2 deficient stromal cells are capable to secrete high amounts of IFNγ which prevents B cell maturing and leads to enhanced B cell apoptosis[62].

All this data emphasize the central role of PTPN2 in many different cell types and also the diversity of cellular processes that depend on this phosphatase.

4.4 Endoplasmic reticulum stress

In addition to the above mentioned processes that connect PTPN2 to IBD, a part of this work centres on the possibility that PTPN2 might contribute to IBD pathogenesis by interfering with ER stress signalling. The connection between PTPN2 and ER stress is already made by a study showing that knockdown of PTPN2 leads to increased ER stress followed by apoptosis in pancreatic β-cells[63]. Due to high secretory activity IECs are as prone to ER stress as β-cells. If there is a PTPN2 deficiency or malfunction this could result in

exaggerated ER stress in IECs, which then could lead to inflammation favouring IBD.

The ER plays a central role in the secretory pathway. Proteins that are destined to be secreted are synthesized by ribosomes attached to the ER membrane. Inside the ER, chaperones assist in correct folding and enzymes add post-translational modifications. Then correctly folded proteins are translocated to the Golgi where they are packed into vesicles and carried to the plasma membrane. The secretion is conducted by fusion of the vesicle with the plasma membrane, thereby expelling the vesicle content in the cell surroundings.

Misfolded proteins are delivered to the cytoplasm and marked for degradation in the proteasome. This mechanism is called ER-associated degradation (ERAD). ER stress occurs when disturbances of the ER homeostasis lead to an accumulation of misfolded proteins. There are three different transmembrane proteins located in the ER membrane, which are able to sense an abnormal increase in misfolded proteins: IRE1, protein kinase RNA-like endoplasmic reticulum kinase (PERK) and activating transcription factor (ATF6) (cf. **Figure 4-5**). Each of these stress sensors then initiates a different signalling cascade. Together these three pathways are called unfolded protein response (UPR) and aim to resolve ER stress by degradation of misfolded proteins and stopping global protein synthesis but permitting chaperon expression.

During ER homeostasis, the chaperon binding immunoglobulin protein (BiP) is bound to the luminal domain those three transmembrane proteins. When more chaperons are needed to assist in protein folding, BiP is released thereby activating stress sensors. This leads to oligomerisation and autophosphorylation

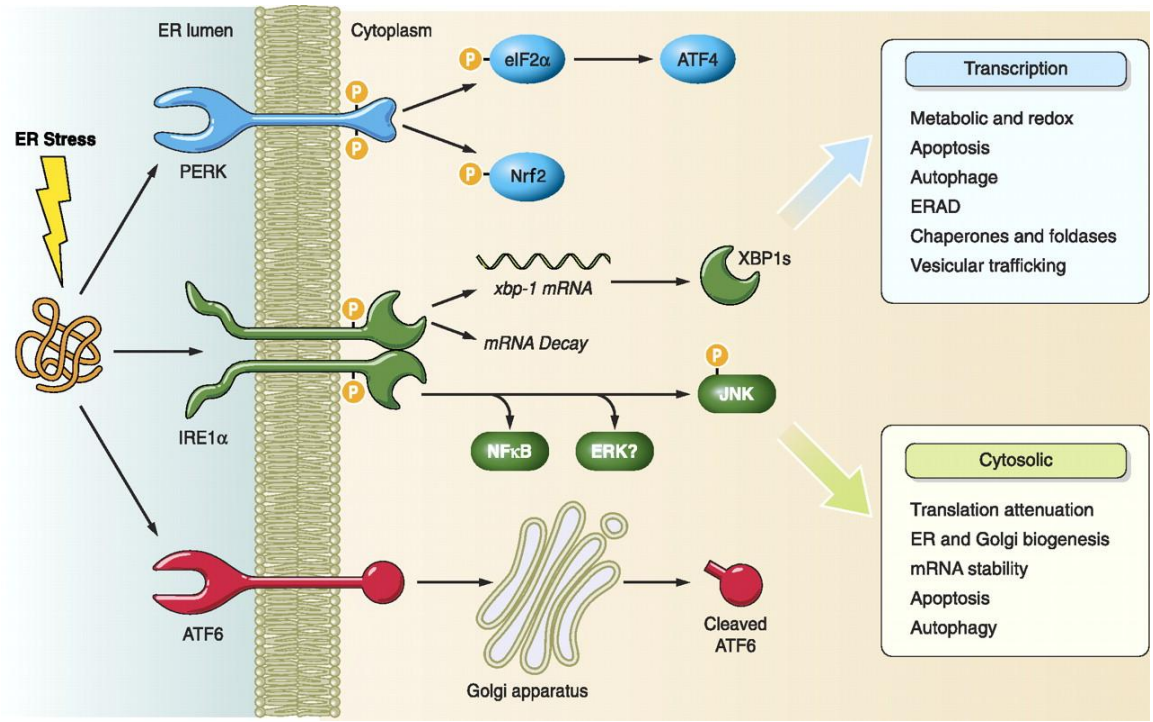

Figure 4-5 The 3 pathways that activate unfolded protein response (UPR) (image source:[64]).There are 3 transmembrane proteins that are able to sense ER stress and each initiates a different signaling cascade: PERK, IRE1α and ATF6. Activated PERK phosphorylates eIF2α and Nrf2. Activated eIF2α increases translation of ATF4 mRNA and ATF4 protein operates as transcription factor, thereby inducing genes involved in amino acid metabolism, autophagy, antioxidant responses and apoptosis. If IRE1α becomes activated by accumulation of unfolded proteins, it controls selective mRNA decay and splicing of XBP1 mRNA. The spliced mRNA can then be translated into XBP1 protein, a transcription factor responsible for upregulation of UPR genes involved in folding, organelle biogenesis, ERAD autophagy and protein quality control. Third ER stress sensor ATG6 is transported to the Golgi upon ER stress induction where a cleavage results in release of the cytosolic domain. This cytosolic ATF6 domain then enters the nucleus and mediates expression of ER chaperons, ERAD-related genes and proteins involved in ER/Golgi expansion.

of the cytosolic IRE1α and PERK domain. IRE1α thereby activates its RNase domain to process X-box binding protein 1 (XBP1) mRNA which then can be translated into protein. XBP1 is a transcription factor that initiates UPR associated genes. Activated PERK has kinase function and phosphorylates eukaryotic initiation factor 2 (eIF2α). EIF2α is necessary for general initiation of translation and is set to inactive state by phosphorylation. This way global protein synthesis is stopped. However, activated ATF6 translocates to the Golgi where it is cleaved. The ATF6 p50 fragment of the cytosolic domain then enters the nucleus and promotes expression of chaperones. If ER stress cannot resolved by these mechanisms the cell undergoes apoptosis.

ER stress is already known to play a role in immunity and inflammation. IRE1α can also be activated via toll-like receptor (TLR) signalling and consequentially, XBP1 can support the expression of pro-inflammatory cytokines in macrophages[65]. In contrast, TLR signalling in macrophages can also prevent ATF6 activation[66]. Furthermore, dysfunctional UPR has been associated with IBD and other autoimmune diseases such as multiple sclerosis and rheumatoid arthritis[67]. As already mentioned, XBP1 is one of the IBD risk genes that plays a role in the ER stress signalling. This goes in line with the finding that tissue specific XBP1 knockout in IECs results in spontaneous enteritis[68]. This inflammation is mediated by Paneth cell dysfunction and IEC's hyper reactivity towards TNF and flagellin. UPR plays a role in tumorigenesis, too. The growth conditions within a tumor typically feature ER stress triggers like hypoxia, low pH

and starvation. Indeed, inhibition of XBP1 protein leads to increased apoptosis, suggesting its importance for tumor survival[69].

As described above, UPR mechanisms play a central role in IBD pathogenesis and it is already shown that PTPN2 interacts with ER stress signalling in β-cells[63]. Thus, it sounds reasonable that PTPN2 mediated susceptibility to IBD is caused by dysfunctional PTPN2 in IEC causing exaggerated ER stress ultimately favouring IBD.

4.5 Models for IBD research

To investigate pathological mechanisms of IBD many *in vitro* and *in vivo* models are available.

4.5.1 *In vitro* models for IBD

The intestinal mucosa contains a large number of different cell types that can affect the epithelium. *In vitro* models allow the isolated examination of a single cell type without complex interaction with surrounding cells and tissues. It permits the complete control over the experimental conditions: cell type, growth/culture conditions and addition of stimulatory molecules are well defined and the over-/under-expression of a protein of interest is easy to facilitate. *In vitro* studies can target potential key cells or mediators in any physiological/pathological process. Additionally, the expression of a particular molecule in the cell type of interest is possible. An array of IEC cell lines derived from colon carcinoma are available. Seeded on filter supports they form a polarized monolayer thereby allowing the investigation of effects of different

stimuli on barrier function and vectorial ion transport. Co-culture of IECs with other cells such as immune cells adds more complexity to the system. It can be used for example to define the cellular interactions that regulate the epithelium. Adding neutrophils to the basolateral compartment can be useful to study transepithelial migration, when a chemoattractant (such as N-formylated peptide) is added apically. It is also possible to study early events and cellular response to a given microbe. However, data obtained from experiments with cell lines have to be interpreted carefully. It needs to be kept in mind that cell lines are immortalized and derive from cancerous tissues, therefore they may not exactly mimic the behaviour of IECs *in vivo*. Immortalization and transformation often alter IEC's specific response to drugs and treatments. Nevertheless*, in vitro* studies are suitable instruments to investigate effect on barrier function, ion transport, absorption mechanisms, injury and repair mechanisms and immune events.

4.5.2 *In vivo* models to study IBD

For *in vivo* studies several mouse models are available. Many transgenic mouse strains exist for example tissue specific knockout and inducible promotors. Some transgenic mouse strains spontaneously develop colitis. The most common one is the IL-10 knockout mouse. The cytokine IL-10 plays a key role in suppression of the immune system by mediating inhibition of T cell activation. It directly acts on APCs and leads to down regulation of MHCII and co-stimulatory molecules and inhibition of IL-12 secretion[70]. IL-10 knockout mice develop colitis by 2-4 month of age with a transmural inflammation and they often develop osteoporosis (as do CD patients) and colon cancer[71]. There are also some other

mouse strains that develop spontaneous colitis such as IL-2 knockout, MHCII knockout or TGFβ knockout mice[72]. A major issue with these models is that onset and severity of colitis are variable and also depend on cleanliness of the animal facility. Germ free mice with IL-10 deficiency do not develop colitis. Obviously, a trigger from microbiota is necessary for the development of spontaneous colitis. Another difficulty with all these models (and with mice experiments in general) is the great importance of the genetic background. For example C57BL/6J IL-10 knockout mice develop only mild colitis whereas a C3H background leads to more severe colitis[73].

Infection models allow pathogen host interaction studies. *Citrobacter rodentium* and *Salmonella typhimurium* are often used in mice that are pre-treated with antibiotics to remove the normal gut microbiota prior infection. However, pathogenesis of murine infections often differs from human: Salmonella infection leads to gastroenteritis in humans but mice get a systemic disease. Early interaction events are specific for every microbe but the late consequences are remarkably uniform: release of chemoattractant cytokines, NF-κB mediated response, IEC apoptosis and altered cell function due to bacterial products.

There are also some chemical inducible models for IBD available. The most common one is the dextran sodium sulphate (DSS) induced colitis. DSS is directly toxic to IECs thereby leading to colon inflammation that resembles UC. This inflammation is accompanied by hyperemia, ulcerations, submucosal edema, lesions, infiltration of granulocytes, bloody diarrhoea and weight loss. DSS can be mixed into the drinking water for 7 days to induce acute colitis. Development of chronic colitis is also possible by administering 4-5 cycles of

DSS dispersed by water treatment. Severity and onset of colitis can be controlled via concentration and duration of DSS treatment. But again, the genetic background and cleanliness greatly influence colitis severity. There are a lot of other chemicals available that are also suitable to induce colitis. The most important ones are trinitrobenzenesulfonic acid (diffuse colonic transmural inflammation) and oxazolone (model for UC). Both chemicals have to be dissolved in ethanol and are applied intrarectally. Furthermore, the DSS colitis can be combined with i.p. injection of azoxymethane (AOM) to induce the development of CRC.

Another possibility to study IBD pathogenesis is the adoptive transfer. Naïve $CD4^+$ T cells from healthy wild type mice are injected into syngenic immunedeficient recipients (RAG knockout or SCID mice) who develop colitis after 5-8 weeks. Transfer colitis mimics the histological features of human UC with crypt abscesses, ulcerations and infiltration in the mucosa. It is a useful method to study the very earliest immunological events, perpetuation of the disease and the role of regulatory T cells. The adaptive transfer experiments are easy to perform, highly reproducible and show many similarities to human IBD. Also bone marrow reconstitution is possible

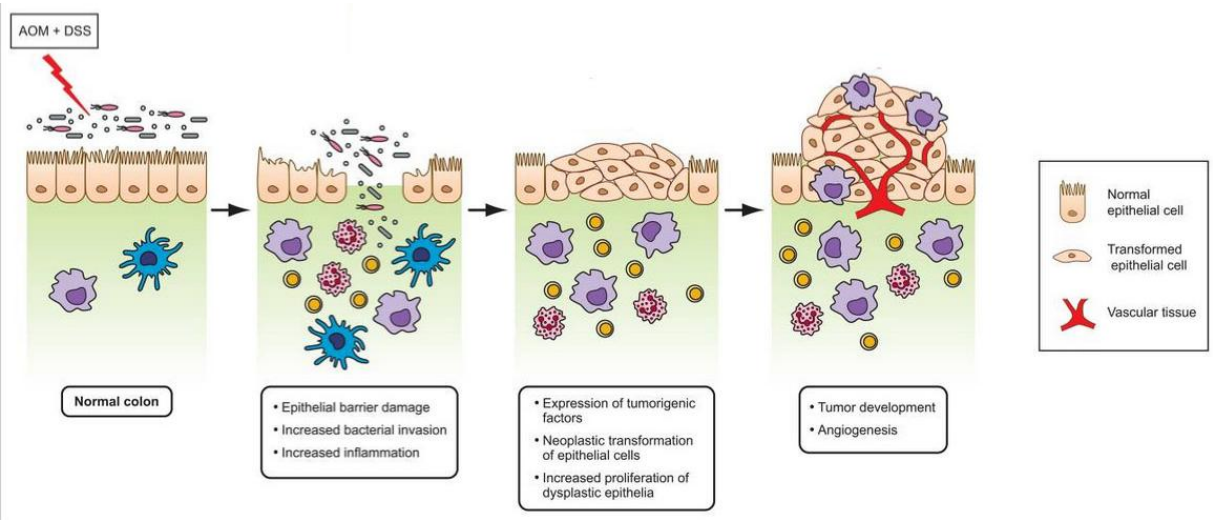

Figure 4-6 Model of DSS colitis and AOM/DSS induced development of colorectal cancer (image source: adapted from [74]).

4.5.3 Mixed models to study IBD

In vivo studies are cost intensive and only limited data end points are possible because mice have to be killed to remove the colon for detailed analysis. Long term culture of isolated intestinal crypts or intestinal stem cells[75] combines the advantages from both *in vitro* and *in vivo* studies. Supplemented with growth factors and three dimensional extracellular matrix these stem cells develop into organoids and enteroids. This is useful to study intestinal stem cell self-renewal, growth and differentiation. *In vitro* expanded organoids may be used for stem cell therapy in preclinical animal models[76] or to study toxicity and cell death induction. Furthermore, organoids from transgenic mice can be used to study the impact of certain proteins.

Xenocraft models allow the performance of experiments with human tissue by transplanting biopsies of human intestine s.c. into immunodeficient mice (SCID or $Rag^{-/-}$). After initial degradation the graft begins to regrow small segments

after 10 weeks. Additionally, the graft can be injected with microbes or their products. But this method has its limits: for example adoptive cell transfer is not possible because human myeloid cells cannot invade graft when vascularisation is of mouse origin.

5 OBJECTIVES

5.1 Aim of the project and addressed topics

GWAS found PTPN2 to be associated with IBD (also to diabetes type 1 and rheumatoid arthritis). When starting the project, it was already known that PTPN2 interferes with ER stress signalling in pancreatic β-cells and that PTPN2 deficiency in IECs leads to barrier defects and impaired autophagy. Therefore, it was a promising approach study the role of PTPN2 in ER stress signalling in IECs and monocytes, two cell types that are heavily involved in IBD pathogenesis. There are some other known ER stress modulators among the IBD susceptibility genes, so this could reveal the mechanism by which PTPN2 mediates predisposition to IBD.

Already available *in vivo* data showed that total loss of PTPN2 is lethal. Mice with a global PTPN2 knockout die few weeks after birth on systemic inflammation. To look at the role of PTPN2 specifically in IECs we generated mice with a tissue specific knockout. We expected these PTPN2xVilCre mice to be more susceptible to DSS induced colitis.

Within this project, following topics were addressed:

- *in vitro*: impact of PTPN2 knockdown on ER stress in THP-1 and HT-29 cells

- *in vivo*: effect of tissue specific PTPN2 knockout in IECs on DSS induced colitis

5.2 Contribution to further projects during the time of the dissertation (not addressed in detail within this work)

5.2.1 PTPN2 controls differentiation of CD4+ T cells and limits intestinal inflammation and intestinal dysbiosis

Loss of PTPN2 in the T cell compartment causes enhanced induction of Th1 and Th17 cells, but impaired induction of regulatory T cells in several mouse colitis models, namely acute and chronic dextran sodium sulfate colitis, and T cell transfer colitis models. This results in increased susceptibility to intestinal inflammation and intestinal dysbiosis which is comparable with that observed in CD patients. We detected inflammatory infiltrates in liver, kidney, and skin and elevated autoantibody levels indicating systemic loss of tolerance in PTPN2 deficient animals. CD patients featuring a loss-of-function PTPN2 variant exhibit enhanced Th1 and Th17 cell, but reduced regulatory T cell markers when compared with PTPN2 wild type patients in serum and intestinal tissue samples. Our findings indicate a novel and crucial role for PTPN2 in chronic intestinal inflammation.

Spalinger MR*, **Kasper S**, Raselli T, Frey-Wagner I, Gottier C, Lang S, Atrott K, Vavricka SR, Mair F, Becher B, Lacroix C, Fried M, Rogler G, Scharl M

Mucosal Immunology 2014, [Epub ahead of print]

doi:10.1038/mi.2014.122.

5.2.2 Activation of protein tyrosine phosphatase non-receptor type 2 by spermidine exerts anti-inflammatory effects in human THP-1 monocytes and in a mouse model of acute colitis

Activation of PTPN2 by spermidine ameliorates IFNγ induced inflammatory responses in THP-1 cells. Furthermore, spermidine treatment significantly reduces disease severity in mice with DSS-induced colitis; hence, spermidine supplementation and subsequent PTPN2 activation may be helpful in the treatment of chronic intestinal inflammation such as IBD.

Moron B*, Spalinger M**, Kasper S**, Atrott K, Frey-Wagner I, Fried M, McCole DF, Rogler G, Scharl M

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6 FIRST MANUSCRIPT

A Cell Type specific Role of The Protein Tyrosine Phosphatase Non-Receptor Type 2 in Regulating ER Stress Signalling

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- ELISAs (SHK)
- AnnexinV staining, FACS analysis (SHK, MRS, TR)
- Manuscript writing (SHK, MS)
- Figure design (SHK, MS)

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A Cell Type-Specific Role of Protein Tyrosine Phosphatase Non-Receptor Type 2 in Regulating ER Stress Signalling

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Key Words
Inflammatory bowel disease · PTPN2 · ER stress

Abstract
Background/Aims: Genetic polymorphisms within the gene locus encoding protein tyrosine phosphatase non-receptor type 2 (PTPN2) have been associated with inflammatory bowel disease (IBD). A recent study demonstrated that PTPN2 regulates ER stress signalling in pancreatic β-cells. Therefore, we investigated whether PTPN2 regulates ER stress pathways, apoptosis and cytokine secretion in human intestinal epithelial cells (IECs) and monocytes. ***Methods:*** THP-1 and HT-29 IECs were stimulated with 2 μg/ml tunicamycin (TNM) for the indicated periods of time. For knockdown experiments, cells were transfected using a mixture of three different PTPN2-specific siRNA oligonucleotides. Cell lysates were analysed by Western blot and real-time PCR. Cytokine secretion was studied by ELISA measurements of cell culture supernatant. ***Results:*** TNM treatment reduced PTPN2 protein levels in HT-29 IECs and THP-1 monocytes. Knockdown of PTPN2 in THP-1 monocytes led to an exaggerated induction of phospho-eIF2α, enhanced PARP cleavage, indicative of apoptosis, and attenuated IL-8 and TNF secretion upon TNM stimulation. In HT-29 cells PTPN2 deficiency caused reduced phosphorylation of eIF2α and PARP cleavage under ER stress conditions. ***Conclusions:*** Whereas the knockdown of PTPN2 made THP-1 cells more susceptible to ER stress, PTPN2 deficiency reduced ER stress responses in HT-29 IECs. This suggests that PTPN2 regulates adaptation to ER stress in a cell type-specific manner.

Introduction

Inflammatory bowel diseases (IBD), a group of chronic inflammatory conditions of the colon and the small intestine, are rampant in industrialized nations [1]. The main types of IBD are Crohn's disease (CD) and ulcerative colitis (UC) [2]. Both genetic and environmental factors contribute to the pathogenesis of IBD. The current hypothesis suggests that a defect in the intestinal epithelial barrier results in an over-activation of the immune system in response to the commensal flora that ultimately leads to chronic intestinal inflammation [3]. Several genome-wide association studies revealed numerous gene loci that mediate susceptibility to IBD [4]. Among them is the locus encoding the protein tyrosine phosphatase non-receptor type 2 (PTPN2) (also called T cell protein tyrosine phosphatase (TCPTP)) [5, 6], which was also found to be associated with other chronic inflammatory and autoimmune diseases such as type 1 diabetes (T1D) and rheumatoid arthritis (RA) [7, 8].

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Some IBD susceptibility loci are also associated with endoplasmic reticulum (ER) stress [4]. ER stress-induced inflammation plays a central role in the pathogenesis of many metabolic, airway and intestinal diseases [9]: it is an important factor in the development of cystic fibrosis (CF) and provides the link between obesity and type 2 diabetes (T2D) [9]. ER stress arises when unfolded or misfolded proteins accumulate in the ER, thus initiating the unfolded protein response (UPR) and limiting further translational events. The accumulation of un- and misfolded proteins can be sensed by three different transmembrane proteins located in the ER membrane that collaborate to minimize the cellular protein load. These sensor proteins, namely protein kinase RNA-like endoplasmic reticulum kinase (PERK), inositol-requiring enzyme 1 (IRE1) and activating transcription factor 6 (ATF6), subsequently initiate specific signalling cascades that ultimately result in the activation of the UPR [10]. If ER stress persists, the UPR triggers apoptosis and local inflammation [11]. Deregulated ER stress responses have been shown to play a critical role in the onset of intestinal inflammation [12].

During CD in particular, an increased rate of intestinal epithelial cell (IECs) apoptosis can be observed and critically contributes to an increased passage of ions and water as well as of bacterial and food antigens across the intestinal epithelium [13–15]. These apoptosis-related barrier defects can be induced by tumor necrosis factor (TNF) in vitro [16], while the extent of TNF-induced apoptosis is potentiated by co-administration of interferon gamma (IFN-γ) [17]. In contrast, a decreased number of apoptotic events in inflammatory cells, such as lymphocytes or monocytes, located in the intestinal lamina propria has been observed in IBD, thus contributing to an overwhelming inflammatory state [18, 19].

In a recent study, PTPN2 was shown to be critically involved in ER stress signalling in pancreatic β-cells [20], whereby the induction of ER stress resulted in a decline in PTPN2 expression. On the other hand, knockdown of PTPN2-alleviated tunicamycin (TNM)-induced ER stress and protected the cells from ER stress-induced apoptosis. Furthermore, the study showed that PTPN2 mainly regulates the PERK/eukaryotic initiation factor 2α (eIF2α) branch of the ER stress signalling pathways [20]. We have previously shown that PTPN2 expression is altered during acute inflammation in CD and UC [21] and further that PTPN2 regulates cytokine secretion and apoptosis in human IECs and monocytes [22, 23].

Therefore, the aim of our study was to investigate whether PTPN2 might be a key regulator of ER stress in human monocytes and intestinal epithelial cells. We demonstrate through this study that PTPN2 controls the PERK/eIF2α pathway of the UPR resulting in altered levels of apoptosis and secretion of pro-inflammatory cytokines. Worthy of note is that these effects were clearly dependent on a specific cell type.

Materials and Methods

Cell Culture and Stimulation Protocols

Human monocytic THP-1 cells (Sigma-Aldrich, St. Louis, Mo., USA) were cultured in RPMI 1640 medium (Life Technologies, Gibco, Carlsbad, Calif., USA) with additional 10% fetal calf serum (FCS) and were maintained in a 5% CO_2 and 95% humidified incubator at 37°C. Cells were seeded 5 h prior to experiment in 1 ml of FCS-free RPMI 1640 medium at 1×10^6 cells/ml per well. Human intestinal epithelial cells HT-29 (ACC 299, DSMZ, Braunschweig, Germany) were cultured in DMEM medium (Life Technologies, Carlsbad, Calif., USA) with additional non-essential amino acids solution (Life Technologies, Gibco) and 10% FCS and were maintained at 37°C in an incubator with 10% CO_2 and 95% humidity. Cells were seeded for 24 h and transferred into FCS-free medium 5 h prior to experiments. TNM (obtained from Sigma Aldrich and dissolved in DMSO) was administered in a concentration of 2 μg/ml for the indicated periods of time.

Preparation of Whole Cell Lysates

Cells were washed twice with phosphate buffered saline (PBS) and lysed in M-Per Mammalian protein extraction reagent (Pierce Biotechnology, Rockford, Ill., USA) supplemented with protease inhibitors (Roche, Basel, Switzerland) for 30 min on ice. After centrifugation (10 min at 13,000 *g*), cell lysate supernatants were assayed for protein content using a NanoDrop spectrophotometer (NanoDrop ND1000; Pierce Biotechnology).

Western Blotting

Each lysate was mixed with NuPAGE® 4× LDS Sample Buffer (Life Technologies) loading buffer, 500 mM dithiothreitol (DTT) and boiled for 5 min at 95°C. Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes (Millipore, Billerica, Mass., USA). Membranes were blocked during 1 h with blocking solution (3% milk powder (C. Roth GmbH + Co. KG, Karlsruhe, Germany) and 1% bovine serum albumin (BSA) (GE Healthcare, PAA Laboratories GmbH, Pasching, Austria)) in aqua bidest. Primary antibodies [monoclonal mouse anti-human PTPN2 antibody was obtained from Merck Millipore; monoclonal rat anti-chicken gp96 antibody from Enzo Life Sciences; polyclonal rabbit anti-human eIF2α, monoclonal rabbit anti-human phospho-eIF2α (Ser^{51}) and polyclonal rabbit anti-PARP were obtained from Cell Signaling Technologies (Danvers, Mass., USA)] were diluted in blocking solution (1:1,000 for all experiments). For phospho-eIF2α detection we used 3% BSA in bi-distilled water for blocking and antibody incubation. Membranes were incubated in primary antibody solution overnight at 4°C and then washed with washing buffer (Tris buffered saline containing 1% Tween 20) for 30 min. Horseradish peroxidase (HRP)-labelled secondary anti-mouse- or anti-rabbit-IgG-antibody (1:5,000; Santa Cruz Biotechnologies, Santa Cruz,

Calif., USA) in blocking solution was added for 1 h and membranes were washed again for 30 min. Immunoreactive proteins were detected using an enhanced chemiluminescence detection kit (Thermo Scientific, Rockford, Ill., USA).

RNA Isolation and Complementary DNA Synthesis

THP-1 cells were washed with ice-cold phosphate buffered saline (PBS) and disrupted in RLT buffer (Qiagen, Venlo, the Netherlands) and 1 M DTT solution. Total RNA was isolated using RNeasy Plus Mini Kit (Qiagen) according to the manufacturer's instructions. RNA concentration was measured by absorbance at 260 and 280 nm (NanoDrop ND1000). Complementary DNA (cDNA) synthesis was performed using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, Calif., USA) following the manufacturer's instructions.

Real-Time Polymerase Chain Reaction

Real-time polymerase chain reaction (PCR) was performed using FAST qPCR MasterMix for Taqman Assays (Applied Biosystems) on a Fast 7900HT Real-Time PCR system using SDS Software (Applied Biosystems). Measurements were performed in triplicates, human β-actin was used as an endogenous control, and results were analysed by the ΔΔCT method. The real-time PCR was comprised of 45 cycles consisting of a denaturing (95°C, 20 s) and an annealing/extending (60°C, 20 s) step. Gene expression assays were all obtained from Applied Biosystems.

Small Interfering RNA (siRNA) Transfection

Three different annealed silencer predesigned siRNA oligonucleotides targeting the ptpn2 gene were obtained from Life Technologies Ltd. Per transfection, 100 pmol of each of the three gene-specific siRNA oligonucleotides were transfected into 1×10^6 THP-1 or HT-29 cells using the Amaxa nucleofector system (Lonza, Walkersville, Md., USA) or 100 pmol of Silencer Negative Control No. 1 siRNA (life technologies) according to the manufacturer's instructions resulting in a final siRNA concentration of 1 nmol/ml. After transfection, cells were cultured in 1 ml medium for 36 h before treatment.

Enzyme-Linked Immunosorbent Assay (ELISA)

Supernatant of THP-1 and HT-29 cells were collected and stored at –20°C. ELISA kits detecting human IL-8 and human TNF were obtained from Qiagen. Assays were carried out according to manufacturer's recommendations and absorbance was detected at 450 nm on a BioTek-Synergy Luminescence Reader using Gen 5.1.1.1 Software. Measurements were performed in duplicates.

Annexin V Staining

Cells were collected, washed in PBS, transferred into 190 µl FACS binding buffer (10 mM HEPES/NaOH pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$), 5 µl annexin V-APC antibody (Enzo Life Science) and 5 µl propidium iodide (Fluka) were added. After 10 min incubation at room temperature in the dark, cells were washed in PBS and resuspended in 200 µl FACS binding buffer. Analysis of apoptosis was done using FACS-Canto II flow cytometry.

Statistical Analysis

Statistical analysis was performed using ANOVA and Student Newman Kels post-hoc test using GraphPad Software (Instat, San Diego, Calif., USA).

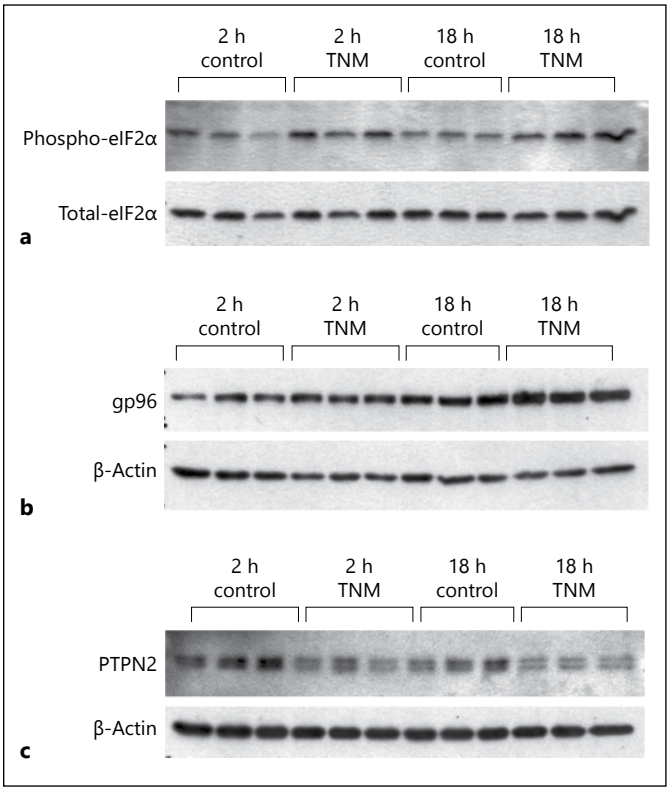

Fig. 1. ER stress decreases levels of PTPN2 protein in THP-1 cells. THP-1 cells were treated with TNM (2 µg/ml) over the indicated period of time to trigger ER stress. Western blots show levels of (**a**) phosphorylated and total eIF2α, (**b**) gp96, (**c**) PTPN2 and the loading control β-actin. Experiments were performed in triplicates.

Results

ER Stress Reduces PTPN2 Protein Levels in THP-1 Cells

To study the possible involvement of PTPN2 in ER stress signalling in human THP-1 monocytes, we treated THP-1 cells with TNM to induce ER stress. The cells were stimulated with 2 µg/µl TNM for 2 and 18 h and lysates were analysed by Western blot for PTPN2 expression and for ER stress-associated proteins. As expected, TNM stimulation led to enhanced eIF2α phosphorylation (fig. 1a) and increased protein levels of the ER chaperon gp96 (fig. 1b), indicative of the induction of ER stress. The ER stress induction was accompanied by decreased levels of PTPN2 protein (fig. 1c).

ER Stress Leads to Changes in Cytokine Expression and Apoptosis in THP-1 Cells

We next investigated to what extent apoptosis and cytokine expression are affected by TNM treatment in

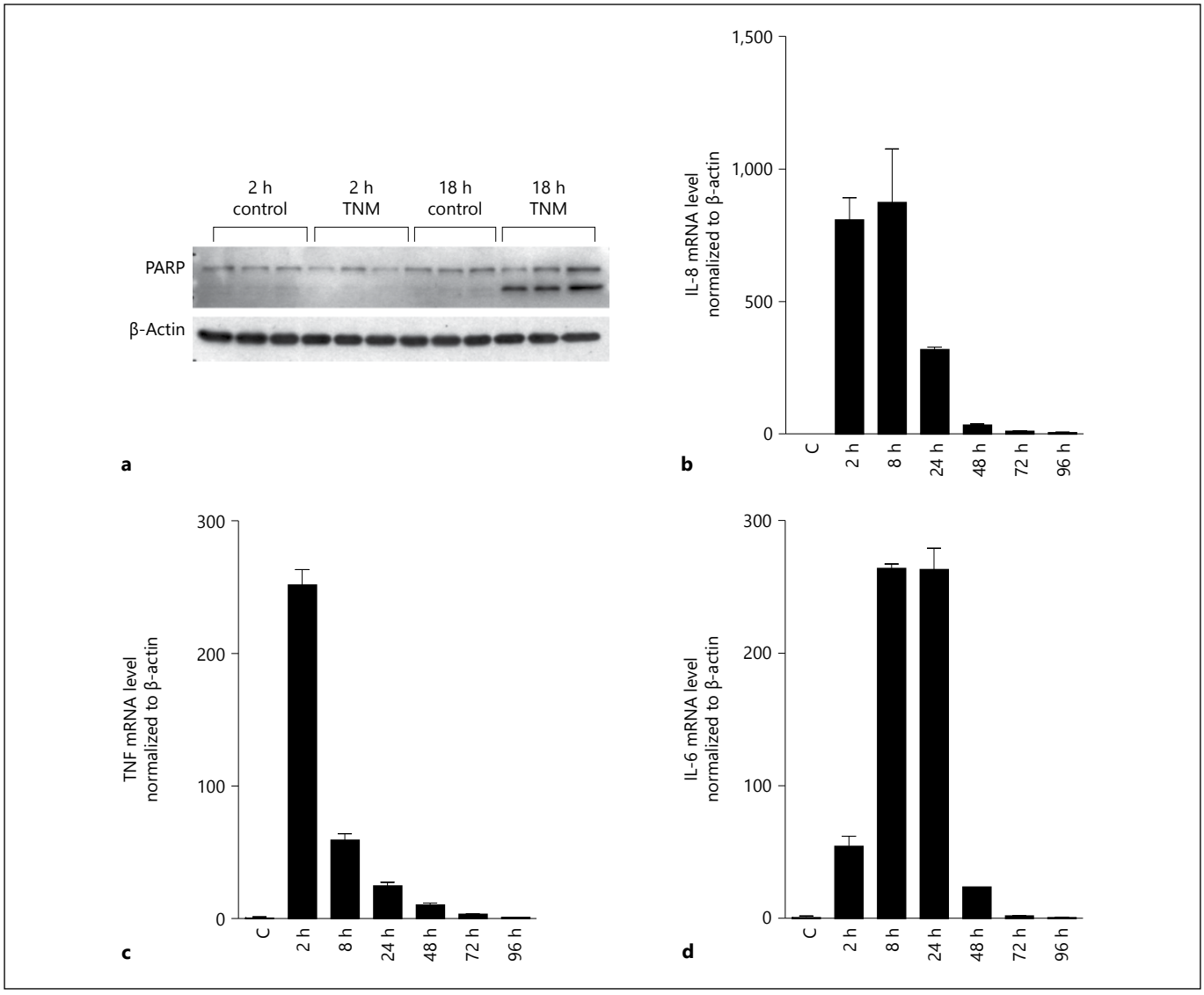

Fig. 2. ER stress increases apoptosis and induces expression of pro-inflammatory cytokines in THP-1 cells. THP-1 cells were treated with TNM (2 μg/ml) over the indicated period of time to trigger ER stress. **a** Western blot shows levels of uncleaved and cleaved PARP and the loading control β-actin. The graphs show mRNA levels of the pro-inflammatory cytokines (**b**) IL-8, (**c**) TNF and (**d**) IL-6. Experiments were performed in triplicates. Data are presented as means ± SD.

THP-1 cells. Therefore, we analysed the lysates (obtained as mentioned earlier) for poly(ADP-ribose)-polymerase (PARP) cleavage by Western blotting. As seen in figure 2a, THP-1 cells showed a strong induction of PARP cleavage after 18 h of TNM stimulation, a sign of apoptosis. To study cytokine expression, we decided to focus on the pro-inflammatory cytokines interleukin 6 (IL-6), IL-8 and tumor necrosis factor (TNF). All three cytokines are known to be increased in IBD patients. Furthermore, anti-TNF antibodies are a widely used treatment in IBD therapy, whereas IL-6 could also be a potential target for future therapeutics. IL-6 antibodies are currently used in clinical studies [24]. Cell lysates were analysed by real-time PCR and the levels of IL-6, IL-8 and TNF mRNA were normalised to the housekeeping gene, β-actin. IL-8 mRNA reached a peak after 8 h of incubation (850× compared to control; fig. 2b). The strongest TNF expression (250× compared to control; fig. 2c) was already detected after 2 h of TNM treatment and declined constantly thereafter. There was also a strong induction of IL-6 mRNA expression after treatment for 8 and 24 h (260× compared to control; fig. 2d).

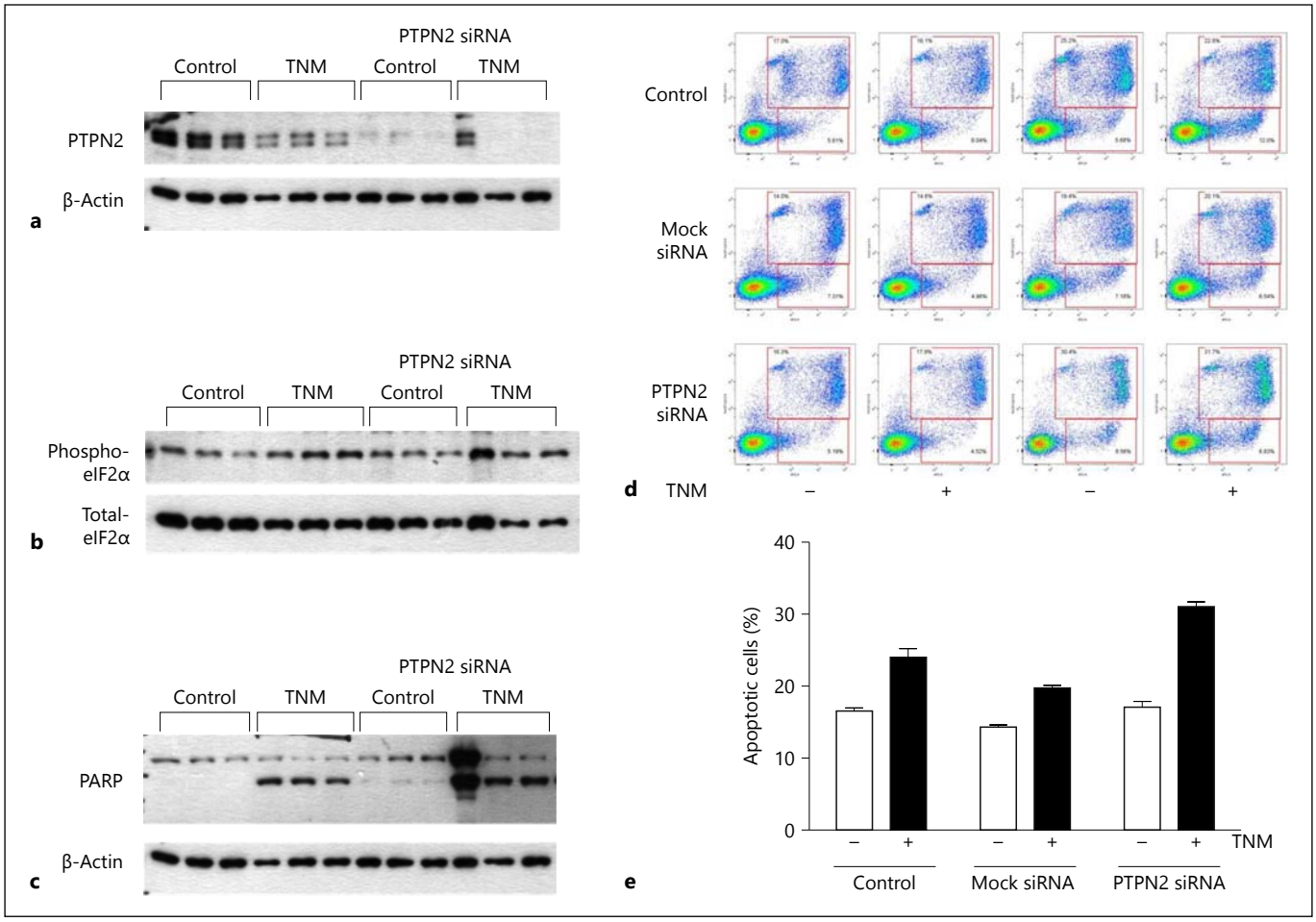

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Fig. 3. PTPN2 knockdown increases ER stress and apoptosis in THP-1 cells. THP-1 cells were transfected with PTPN2-specific or mock siRNA and treated with TNM (2 μg/ml) for 18 h. Western blots show levels of (**a**) PTPN2, (**b**) phosphorylated and total eIF2α, (**c**) uncleaved and cleaved PARP and the loading control β-actin. Experiments were performed in triplicates. FACS analysis of annexin V/PI stained cells (**d**) was used to determine level of apoptosis and graphs shows percentage of double positive cells (**e**). Experiments were done in duplicates.

PTPN2 Knockdown Affects ER Stress Susceptibility in THP-1 Cells

To assess whether PTPN2 is important for regular ER stress responses in THP-1 cells, we knocked down PTPN2 using specific siRNA constructs and treated the cells with TNM for 18 h. Western blotting was performed to analyse the protein content of the cell lysates. The amount of detected PTPN2 protein was strongly reduced after transfection with PTPN2-specific siRNA compared to non-transfected control cells (fig. 3a), indicating that the knockdown was highly effective. In control cells, ER stress reduced PTPN2 protein levels (fig. 3a) as already shown in previous experiments (fig. 1c). PTPN2 deficiency affected phosphorylation of eIF2α under ER stress conditions (fig. 3b), as we observed an exaggerated induction of phospho-eIF2α in those cells. EIF2α phosphorylation levels in unstressed conditions were not dependent on PTPN2 knockdown.

PTPN2 Knockdown Affects Apoptosis and Cytokine Secretion Under ER Stress Conditions

As seen in the previous experiments, TNM stimulation led to a strong increase in PARP cleavage (fig. 3c). In THP-1 cells with downregulated PTPN2 expression upon siRNA transfection, we already observed some PARP cleavage in un-stimulated cells. Treatment with TNM further increased PARP cleavage, which finally reached a higher level than in TNM-treated PTPN2-competent cells. To prove that the observed increase in apoptosis is not secondary to the transfection procedure

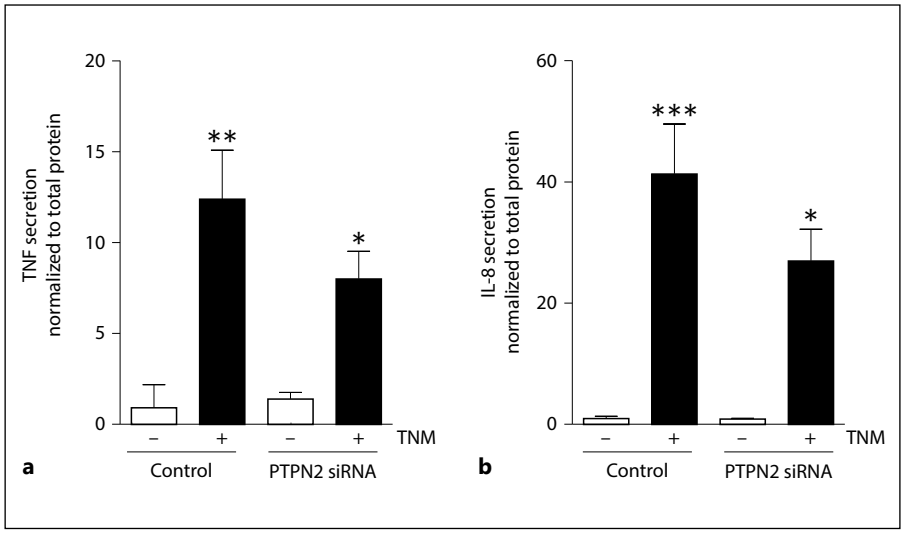


Fig. 4. PTPN2 knockdown impairs pro-inflammatory cytokine secretion in THP-1 cells. THP-1 cells were transfected with PTPN2-specific siRNA and treated with TNM (2 μg/ml) for 18 h. The graphs show secretion of the pro-inflammatory cytokines (**a**) TNF and (**b**) IL-8 measured by ELISA. Data are presented as means ± SD. Experiments were performed in triplicates. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. the respective control.

but is a specific effect of PTPN2 knockdown, we repeated the experiment and used scrambled siRNA as an additional control. FACS analysis of the annexin V/PI stained cells showed no adverse effect of this mock control compared to cells that were not transfected (fig. 3d, e). Furthermore, this experiment confirmed the exaggerated apoptosis of stressed PTPN2-deficient monocytes. To study whether cytokine secretion is also affected by the knockdown of PTPN2, we performed an ELISA analysis to detect the pro-inflammatory cytokines TNF and IL-8. In control cells ER stress induction led to an increase in the secretion of TNF (12× induction; fig. 4a) and IL-8 (40× induction; fig. 4b). In PTPN2 knockdown cells, TNM-induced secretion of both cytokines was strongly attenuated.

HT-29 Intestinal Epithelial Cells Show Only Minor ER Stress Responses

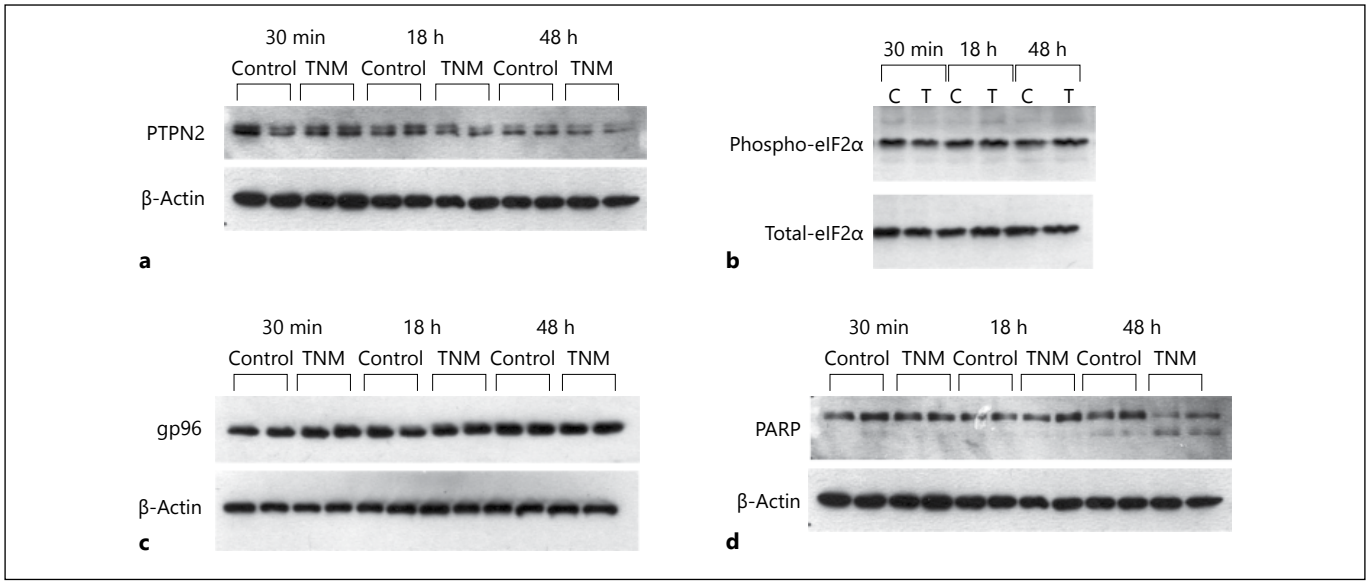
To answer the question whether the observed effects are specific to cell type, we performed experiments using the intestinal epithelial cell (IECs) line HT-29. In a first experiment we used TNM to induce ER stress for 30 min, 18 and 48 hours and analysed cell lysates by Western blotting. TNM stimulation had only minor effects on PTPN2 protein levels, leading to a slight reduction visible mainly at the later time points (fig. 5a). The Western blot for the ER stress marker phospho-eIF2α also showed an induction after 48 h of TNM stimulation, whereas gp96 protein expression did not show any obvious changes upon TNM treatment (fig. 5b, c). Enhanced PARP cleavage as a marker for apoptosis was observed only after 48 h of TNM stimulation (fig. 5d).

PTPN2 Knockdown is Beneficial for HT-29 Cells

To address the role of PTPN2 with respect to ER stress responses in HT-29 IECs we transfected the cells with PTPN2-specific siRNA. ER stress was induced by TNM treatment for 48 h. Cell lysates were analysed by Western blot, and cell culture supernatant was tested for cytokine secretion using ELISA. Figure 6a shows that PTPN2 protein levels were strongly reduced in PTPN2 siRNA transfected cells, suggesting that the knockdown was effective. In control cells, TNM stimulation led to an increase in phospho-eIF2α, which was ameliorated in PTPN2 siRNA transfected cells (fig. 6b). TNM-induced PARP cleavage was higher in control cells than in PTPN2 siRNA-transfected cells. Also, the level of cleaved PARP in untreated cells was reduced in PTPN2 siRNA cells compared to PTPN2-competent cells (fig. 6c). ELISA analysis showed that the cytokine secretion was also affected by PTPN2 knockdown. IL-8 secretion was not changed by TNM treatment in HT29 IECs but was strongly reduced in cells featuring the PTPN2 knockdown (fig. 6d). The baseline secretion of TNF was weakly reduced after knockdown of PTPN2 (fig. 6d). Upon TNM treatment we noted a robust induction of TNF; however, it remained unaffected by the loss of PTPN2.

Discussion

In this study we demonstrated that PTPN2 influences ER stress signalling in the monocytic cell line THP-1 and in HT29 IECs. ER stress induction using TNM resulted in a decline of PTPN2 protein expression in THP-1 cells,



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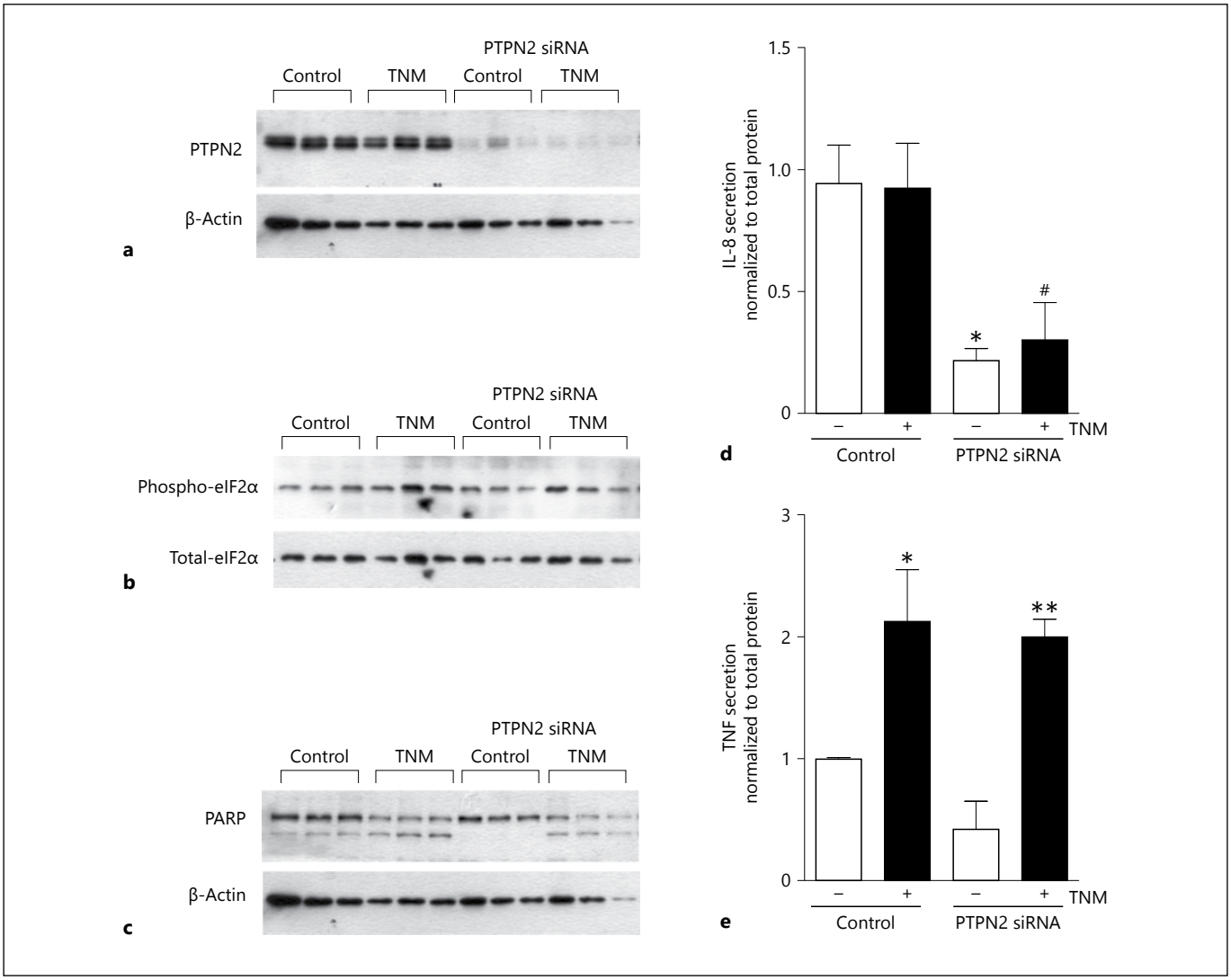

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(*For legend 5 and 6 see next page.*)

thus confirming the previous data of Bettaieb et al., who observed the same effect in pancreatic β-cells [20]. Contrary to their findings in pancreatic MIN6 cells, our study showed that knocking down PTPN2 in THP-1 monocytes gives rise to a higher susceptibility to ER stress: TNM-induced phosphorylation of eIF2α, PARP cleavage and annexin V/PI staining were increased in PTPN2-deficient cells. The TNM-induced expression of the pro-inflammatory cytokines TNF and IL-8 was reduced upon transfection with PTPN2 siRNA. This is possibly due to the inhibition of mRNA translation as a result of UPR activation. Transfecting THP-1 cells with mock siRNA did not have any adverse effects, verifying that our observations are specific to the knockdown of PTPN2.

PTPN2 clearly interacts with the PERK/eIF2α pathway in THP-1 cells. However, we did not formally test the other two arms of the UPR. Further experiments are needed to see whether the other sub-pathways (via IRE1 and ATF6) are also influenced by PTPN2 in these cell types. Nevertheless, Bettaieb et al. have previously shown that the PTPN2-mediated effects on ER stress signalling exclusively affect the PERK/eIF2alpha arm [20]. It is nonetheless evident that the role of PTPN2 in ER stress signalling in THP-1 cells differs from that in MIN6 cells. These are completely different cell types and may have developed in distinct ways to adapt to ER stress. This could be plausible since there are already other proteins known to impact ER stress signalling in a cell type-specific manner [25].

Contrary to our findings in THP-1 monocytes, we observed only minor effects of ER stress on PTPN2 in the colonic epithelial cell line HT-29, namely a minor trend toward declined expression. The effect of PTPN2 deficiency in HT-29 cells differed clearly from our results in THP-1 cells. In HT-29 cells, the knockdown of PTPN2 had beneficial consequences: un-stimulated cells with PTPN2 deficiency showed less apoptosis and less secretion of TNF and IL-8. This seems surprising at first glance, since PTPN2 has been identified as an IBD susceptibility gene. However, reduced levels of secreted cytokines in PTPN2-deficient cells might contribute to a re-balancing of the overwhelming immune response that can be observed in IBD. It must be noted that we did not formally prove that altered apoptosis and cytokine secretion in TNM-treated cells are exclusively dependent on the induction of ER stress. Therefore, there is also the possibility that these events are rather associative than causally linked. However, our data clearly demonstrate that these events are regulated by the action of PTPN2 in the setting of ER stress. In any way, our experiments confirm that the role of PTPN2 in ER stress signalling is dependent on a specific cell type.

In summary, our data demonstrate that PTPN2 regulates the ER stress responses in a cell type-specific manner. While dysfunction of PTPN2 in monocytes might be associated with increased cell death, the loss of PTPN2 in IECs seems to protect the cells in some way. These observations might contribute to a better understanding of the complex role of IBD susceptibility genes in disease pathogenesis and support the observation that PTPN2 is a crucial regulator of ER stress responses.

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Disclosure Statement

The authors declare there are no competing interests.

Author contributions: S.H.K., M.R.S. and T.R. performed experiments and analyzed data. M.S. conceived the experimental study and supervised the project. All authors wrote, corrected and approved the manuscript.

Fig. 5. HT-29 cells show minor ER stress response. HT-29 cells were treated with TNM (2 μg/ml) over the indicated period of time to trigger ER stress. Western blots show levels of (**a**) PTPN2, (**b**) phosphorylated and total eIF2α, (**c**) uncleaved and cleaved PARP, (**d**) gp96 and the loading control β-actin. Experiments were performed in duplicates.

Fig. 6. PTPN2 knockdown is beneficial for HT-29 cells. HT-29 cells were transfected with PTPN2-specific siRNA and treated with TNM (2 μg/ml) for 48 h. Western blots show levels of (**a**) PTPN2, (**b**) phosphorylated and total eIF2α, (**c**) uncleaved and cleaved PARP and the loading control β-actin. The graphs show secretion of the pro-inflammatory cytokines (**d**) IL-8 and (**e**) TNF measured by ELISA. Data are presented as means ± SD. Experiments were performed in triplicates. * Indicates $p < 0.05$, ** indicates $p < 0.01$, vs. the respective control. # Indicates $p < 0.05$ vs. TNM-treated control siRNA transfected cells.

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7 SECOND MANUSCRIPT

Deficiency of Protein Tyrosine Phosphatase Non-Receptor Type 2 in Intestinal Epithelial Cells Has No Appreciable Impact on Inflammation in DSS Colitis

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Contribution to the manuscript:

- Animal breeding (AT, SHK)
- Application for animal license (IFW, MRS, MS)
- Animal care and recording of weight curves (SHK)
- Sacrifice mice and sampling (SHK, IL, AG, TR, CG, KA)
- Colonoscopy (MRS, SHK)
- Determination of murine endoscopic index of colitis severity (MRS, SHK)
- Histological scoring (IL, SHK)
- Isolation of DNA from murine IECs (SHK)
- Study design (MS, SHK)
- Manuscript writing (SHK, MS)
- Figure design (SHK, MS)

ABSTRACT

Background/Aims: Variants in protein tyrosine phosphatase non-receptor type 2 (PTPN2) are known to mediate susceptibility to inflammatory bowel diseases (IBD). Cell culture experiments suggest that PTPN2 influences barrier function, autophagy and secretion of pro-inflammatory cytokines. PTPN2 knockout mice die few weeks after birth on systemic inflammation emphasizing the importance of this phosphatase in inflammatory processes. The aim of this study was to investigate the role of PTPN2 in colon epithelial cells by performing DSS induced colitis in PTPN2xVilCre mice.

Methods: Acute colitis was induced by administering 2.5 or 2.0 % DSS for 7 days and chronic colitis by 4 cycles of treatment using 1 % DSS. The AOM/DSS model was used to study colon cancerogenesis. Body weight of mice was measured regularly and colonoscopy was done at the end of the experiments. Mice were sacrificed afterwards and colon specimens were obtained for H&E staining.

Results: We did not observe significant influence of PTPN2 deficiency on histological scoring for inflammation severity after acute or chronic DSS colitis which was in contrast to differences found in colonoscopy or weight loss. Chronic colitis induced development of aberrant crypt foci more frequently in $PTPN2^{flox/flox}$xVilCre mice compared to their $PTPN2^{flox/flox}$ littermates. AOM/DSS treatment, a model for colon cancer carcinogenesis, led to formation of aberrant crypts in nearly all animals irrespective of genotype.

Conclusions: Loss of PTPN2 in IECs has no significant influence on histological inflammation in DSS colitis. The difference found in colonoscopy scores and weight curves, however, may indicate a functional role of PTPN2 in pathways other than inflammation which has to be further evaluated.

INTRODUCTION

Crohn's disease (CD) and ulcerative colitis (UC) are the most common types of inflammatory bowel diseases (IBD), a group of chronic and relapsing inflammatory conditions of the intestine. Even though the multifactorial pathogenesis is still not entirely understood, it is generally accepted that IBD results from an overrated inflammatory immune response to the otherwise harmless, commensal microbiota in genetically predisposed individuals. Genome-wide association studies (GWAS) helped to identify over 160 susceptibility loci for IBD[1]. Among these loci is the gene encoding protein tyrosine non-receptor type 2 (PTPN2) that has also been reported to be linked to other autoimmune diseases such as diabetes type 1 and rheumatoid arthritis[2-4].

As other phosphatases, PTPN2 is a negative regulator of many signalling pathways. It interferes with cytokine signalling by dephosphorylating some JAK and STAT molecules[5-7] and participates in growth regulation and glucose homeostasis by targeting EGFR and insulin receptor[5]. PTPN2 is a ubiquitously expressed protein found in most mammalian embryonic and adult tissues with higher levels observed in lymphoid cells. In accordance with its potential role as IBD susceptibility gene, PTPN2 expression was found to be increased in intestinal biopsies of CD patients and to a lower extent also in UC patients[8]. Global loss of PTPN2 is lethal: $PTPN2^{-/-}$ knockout mice show defects in haematopoiesis, anaemia, massive tissue infiltration by mononuclear cells and die few weeks after birth[9, 10]. The embryonic development of these mice seems normal, the new-borns are of normal size and health. Nevertheless, at the age of three weeks, they are obviously smaller compared to their wild type littermates

and exhibit hunching, closed eyes, decreased mobility and diarrhea followed by death[9].

From cell culture experiments using intestinal epithelial cell (IEC) lines it is already known that PTPN2 expression is increased by the pro-inflammatory cytokines TNF and IFNγ[8, 11, 12]. Knockdown of PTPN2 potentiates the stimulatory effects of TNF and IFNγ leading to exaggerated epithelial permeability and amplified secretion of interleukin 6 (IL-6) and interleukin 8 (IL-8)[8, 11, 12]. PTPN2 also regulates autophagy: loss of PTPN2 leads to impaired autophagosome formation and dysfunctional autophagy in IECs[13]. Without functional autophagy IECs are more prone to infection by intracellular invaders like *listeria monocytogenes*[13].

The above mentioned *in vitro* findings show the importance of PTPN2 in IECs and the knockout mouse demonstrates that PTPN2 also plays a role in inflammatory processes *in vivo*. Therefore, the investigation of the role of PTPN2 in the intestinal epithelium *in vivo* was a promising approach. To do so, we generated a mouse strain with a tissue specific loss of PTPN2 in IECs. The mice carry loxP sequences flanking exon 3 of the PTPN2 gene and express the Cre-recombinase under the control of the villin promotor. We used these $PTPN2^{flox/flox}$xVilCre mice to perform acute and chronic dextran sulphate sodium (DSS) induced colitis. Regarding the preliminary mentioned data, we expected these mice to suffer from more severe colitis upon DSS treatment.

MATERIALS AND METHODS

Mice and Induction of Colitis

Animal experiments were carried out according to Swiss animal welfare laws and were approved by the veterinary authorities of Zurich, Switzerland (Kanton Zürich Gesundheitsdirektion Veterinäramt, approval no. 54/2011). Due to the approval of the veterinary authorities of Zurich, no further approval by an Institutional Animal Care and Use Committee (IACUC) or ethics committee was necessary. Mice were housed in a specific pathogen-free facility in individually ventilated cages. Food and water were available *ad libitum*. Female PTPN2$^{flox/flox}$ and PTPN2$^{flox/flox}$VilCre littermates on a C57BL/6J background were used for all experiments. Mice with a bodyweight between 20 and 25 g were randomly divided into two DSS and two water control groups. Acute colitis was induced by administering 2.5 % or 2.0 % dextran sulphate sodium (DSS) (MP Biomedicals, Illkirch, France) dissolved in drinking water for 7 days. Chronic colitis was induced by four cycles of treatment with 1.5 % DSS for 7 days interspersed with 10 days of regular drinking water and a final recovery phase of 3.5 weeks. Body weight and disease activity was determined regularly. At the end of the experiment colonoscopy was done and mice were subsequently sacrificed. Spleen and colon were removed, colon length and spleen weight were measured. Colon samples were taken for H&E staining.

DSS/AOM Model to Study Colon Carcinogenesis

4 cycles of DSS administration were combined with repeated intraperitoneal injection of azoxymethane (AOM) (Sigma) (10 µg/g). Each cycle consists of 7 days treatment using 1.5 % DSS followed by 15 days with regular drinking water. AOM injections were done at day 1 of DSS treatment and day 2 of regular

drinking water in each cycle. After 4 cycles mice were allowed to recover for 6 weeks. After every DSS/AOM cycle colonoscopy was performed under isofluran anaesthesia to screen for colon carcinogenesis and potentially end the experiment at an early time point if intense carcinogenesis would have been observed. At the end of the experiment colonoscopy was done and mice were sacrificed to obtain colon samples.

Assessment of Colonoscopy Score in Mice

Animals were anaesthetised intraperitonealy with 90-120 mg/kg body weight ketamine (Vétoquinol, Bern, Switzerland) and 8 mg/kg body weight xylazine (Bayer, Lyssach, Switzerland). Colonoscopy was performed as described previously[14]. Briefly, the solid endoscope was introduced per anus with a lubricant (2 % lidocaine) in the sedated mouse. The colon was gently inflated with air. Recording was performed with the Karl Storz Tele Pack Pal 20043020 (Karl Storz Endoskop, Tuttlingen, Germany). Colonoscopy was scored using the murine endoscopic index of colitis severity (MEICS) scoring system as described previously[85]. In detail, the MEICS score was assessed as follows: The MEICS consists of five parameters: (1) Thickening of the colon wall (transparent, moderate, marked, non-transparent 0-3 points), (2) changes of the vascular pattern (normal, moderate, marked, bleeding 0-3 points), (3) fibrin visible (none, little, marked, extreme 0-3 points), (4) granularity of the mucosal surface (none, moderate, marked, extreme 0-3 points) and (5) stool consistency (normal solid, still shaped, unshaped, spread 0-3 points). This results in an overall score range between 0 and 15.

Assessment of Histological Score in Mice

Histological scoring for inflammatory infiltration and epithelial cell damage was performed on H&E stained section of the most distal 1 cm of the mouse colon as described previously[15]. Score for epithelial damage: 0, normal morphology; 1, loss of goblet cells; 2, loss of goblet cells in large areas; 3, loss of crypts; 4, loss of crypts in large areas. Score for infiltration: 0, no infiltrate; 1, infiltrate around crypt base; 2, infiltrate reaching to L. muscularis mucosae; 3, extensive infiltration reaching the L. muscularis and thickening of the mucosa with abundant oedema; 4, infiltration of the L. submucosae. The total histological score represents the sum of the scores for epithelial damage and infiltration. Histological examination was performed by an independent, blinded investigator.

IEC and DNA isolation from Colon Specimens

After cutting away the most distal 3 cm of colon for other purposes, the remaining part of the colon was sliced lengthwise, freed from residual faeces and cut into small pieces. To loosen IEC, the slices were transferred to Hank's balanced salt solution (HBSS; PAA, Linz, Austria) supplemented with 2 mM of EDTA and stirred for 30 min at 37 °C. Mucus was removed by passing the slurry over a coarse mesh (400 µm; Carl Roth GmbH, Karlsruhe, Germany). The sieve residue was flushed back to the tube with fresh HBSS, vigorously shaken 10 times and vortexed for 1 min. The shake and vortex step was repeated twice resulting in 30 times shaking and 3 min of vortexing. Mucosal pieces were partitioned from detached cells by filtration through mesh. The flow-through was collected and centrifuged at 400 g for 7 min. Supernatant was discarded and the cell pellet was resuspended in PBS. The supernatant was removed and samples

were frozen in liquid nitrogen and stored at -80°C until DNA isolation. The pelleted IECs were defrosted and DNA was isolated using DNA Mini Kit (Qiagen, Düsseldorf, Germany) according to the manufacturer's blood and body fluid spin protocol.

Genotyping

Digestion buffer (25 mM NaOH, 0.2 mM EDTA) was added to ear biopsies and incubated for 1 hour at 98 °C. Equal amount of neutralisation buffer (40 mM TrisHCl, pH 5.5) was added and 1 µl was used for genotyping VilCre PCR to assess the presence of the cre-recombinase. DNA isolated from IECs from colon specimens was used to check if cre-mediated recombination took place via genotyping PCR. Primer sequences VilCre PCR: VilCre_fwd: GTGTGGGACAGAGAACAAACC, VilCre_rev: ACATCTTCAGGTTCTGCGGG, Contr_fwd: CAAATGTTGCTTGTCTGGTG, Contr_rev: GTCAGTCGAGTGCACAGTTT; Primer sequences for PTPN2 recombinant allel: Primer_fwd: GCCAAGAGACAGTGGAAAGAGAG, Primer_rev: ACTGCAAAACCATAACTGGGC

RESULTS

Mice with tissue specific loss of PTPN2 in IECs

To determine the role of the IBD susceptibility gene PTPN2 in the intestinal epithelium *in vivo*, we generated mice that exhibit a tissue specific loss of PTPN2 in IECs ($PTPN2^{flox/flox}$xVilCre mice). To address whether Cre-recombinase mediated recombination of the PTPN2 allele took place two $PTPN2^{flox/flox}$ and two

PTPN2$^{flox/flox}$xVilCre mice were sacrificed and IECs were isolated from colon specimens. DNA was isolated from these IECs and used for genotyping PCR. In fact, Figure 1 shows that Cre-recombinase was active in PTPN2$^{flox/flox}$xVilCre mice and mediated conversion of the PTPN2 allele in IECs.

PTPN2$^{flox/flox}$xVilCre mice tended to suffer more from acute colitis induced by 2.5% DSS

The dextran sulphate sodium (DSS) induced colitis, a model of mucosal inflammation to study IBD pathogenesis, was used for experimental approaches. In a first acute colitis experiment we administered 2.5 % DSS to PTPN2$^{flox/flox}$xVilcre mice and their PTPN2$^{flox/flox}$ littermates for 7 days. The DSS treatment induced severe weight loss in both groups (figure 2a). At the end of the experiment there was no significant difference in weight loss between genotypes but the onset of weight loss was earlier in the DSS treated PTPN2$^{flox/flox}$xVilcre mice compared to their DSS treated PTPN2$^{flox/flox}$ littermates, leading to a significant difference at day three (figure 2a). Colonoscopy at the end of the experiment revealed a significant difference between the DSS groups, with PTPN2$^{flox/flox}$xVilcre mice showing signs of more severe colon damage (figure 2b). DSS treatment led to a shortening of the colon in PTPN2$^{flox/flox}$ and PTPN2$^{flox/flox}$xVilcre mice, but the magnitude was not influenced by PTPN2 deficiency in IECs (figure 2c). Also, the spleen weight was increased by DSS induced inflammation but we did not observe a significant difference between both DSS treated groups (figure 2d). Histological severity of inflammation was scored in H&E stained colon slides with respect to infiltration and epithelial damage. The DSS exposure was clearly reflected by this score, but it was not

further exaggerated in PTPN2$^{flox/flox}$xVilcre animals (figure 2e). There was a very severe inflammation even in the DSS treated PTPN2$^{flox/flox}$ group with one mouse reaching the maximum score of 8. Therefore, it seemed plausible that PTPN2$^{flox/flox}$xVilcre mice did not achieve higher histological scores as severity of inflammation observed in the PTPN2$^{flox/flox}$ animals already was very high. Hence, we decided to lower the DSS concentration in a second acute DSS colitis experiment, expecting to see a more pronounced difference.

PTPN2 deficiency in IECs did not influence inflammation in acute colitis induced by 2% DSS

In a further experiment we used 2.0 % DSS for 7 days to induce acute colitis. The resulting weight curve is shown in figure 3a. No differences between equally treated groups was observed with respect to weight loss during the whole experiment (figure 3a). The early onset of weight loss in the DSS treated PTPN2$^{flox/flox}$xVilcre mice noticed upon 2.5% DSS treatment was no longer observed. Colonoscopy and MEICS score also did not reveal significant differences between genotypes (figure 3b). There was only a higher MEICS score in PTPN2$^{flox/flox}$xVilcre animals. The DSS treatment resulted in shortening of the colon and increased spleen weight but the effect was independent from genotype (figure 3c,d). The histological score in H&E stained colon slides also was not affected by epithelial PTPN2 deficiency (figure 3e).

PTPN2$^{flox/flox}$xVilcre mice tended to suffer less from chronic DSS colitis

To evaluate whether a chronic inflammation would reveal a phenotype in PTPN2$^{flox/flox}$xVilcre mice we performed a chronic colitis experiment by four

cycles of DSS treatment and a final recovery phase of four weeks. No significant difference with respect to the weight curve was observed (figure 4a). In contrast to acute colitis, DSS treated PTPN2$^{flox/flox}$xVilcre animals showed significantly less severe colitis compared to their PTPN2$^{flox/flox}$ littermates during colonoscopy (figure 4d). The difference with respect to colonoscopy findings and MEICS score again was not resembled by analysis of the histological score (figure 5a). A very similar degree of mucosal damage was found in both DSS groups. However, we noticed epithelial abnormalities in DSS treated PTPN2$^{flox/flox}$xVilcre mice that were not observed during acute colitis experiments (figure 5b). Several aberrant crypts of different shapes formed clusters that were easily distinguished from the surrounding tissue. Such aberrant crypt foci are known to be a consequence of exposure to carcinogens and could develop into colon cancer[16, 17]. Aberrant crypt foci were observed more frequently in DSS treated PTPN2$^{flox/flox}$xVilcre mice (figure 5b). In one mouse abnormal foci even penetrated the lamina muscularis and aberrant crypts were found in the submucosa. This could be an indicator for increased colon cancer susceptibility in mice that carry IEC specific PTPN2 deficiency. To address this issue we next performed DSS/AOM colitis, a model widely used to study colon cancer.

PTPN2 deficiency in IECs did not affect colon carcinogenesis in DSS/AOM colitis

We performed DSS/AOM colitis by administering four cycles of DSS combined with repeated AOM injection. Weight indicated no differences between genotypes (data not shown). Colonoscopy showed signs of inflammation but we did not detect any tumors (data not shown). The DSS induced inflammation

resulted in a shortened colon but this was not influenced by IEC PTPN2 deficiency (data not shown). The repeated AOM injection combined with DSS treatment induced aberrant crypt foci in nearly all animals irrespective of genotype (figure 5c).

DISCUSSION

When mice were treated for seven days with 2.5% of DSS an early onset of weight loss in PTPN2$^{flox/flox}$xVilcre mice compared to their PTPN2$^{flox/flox}$ littermates was observed and colonoscopy showed significantly worse colitis in those animals. When treated with 2.0% DSS for seven days these differences did not reoccur. In contrast, in chronic DSS colitis, mice with PTPN2 deficiency in IECs tended to less weight loss and colonoscopy revealed less severe colitis. For all conditions tested histological scoring did not indicate differences between genotypes with respect to epithelial damage and inflammatory infiltrates. Histological scoring is usually regarded as the most solid parameter in colitis experiments. However, the differences found in weight loss or colonoscopy point to the fact that there was a discrepancy between clinical features of disease and histology. In the context of DSS colitis epithelial specific PTPN2 deletion in IECS only plays a minor for the induction of the inflammatory infiltrate but may nevertheless have a role for overall disease severity.

Although cell culture studies were able to show the importance of PTPN2 expression in IECs for barrier function, secretion of pro-inflammatory cytokines and autophagy[8, 11-13] this seems to be less relevant *in vivo*. DSS induced inflammation is mediated by the interplay of many different cells types including IECs and cells of the innate and adaptive immune system. When PTPN2 function is lost in IECs this seems to influence the histological changes only marginally. In contrast, *in vivo* studies using other tissue and/or cell specific PTPN2 knockout mouse strains have already demonstrated the importance of this phosphatase for the differentiation of $CD4^+$ cells[18] but also for TCR signalling

and lymphopenia induced proliferation in $CD8^+$ cells[19, 20]. Apparently, loss of PTPN2 in the T cell compartment leads to stronger phenotypes. This may be explained by the fact that PTPN2 is highest expressed in T cells and was first discovered in a screening of a human T cell cDNA library[21].

After induction of chronic colitis we detected epithelial aberrant crypt foci in the distal colon. This kind of epithelial transformations has been described as a result of exposure to carcinogens and could develop into colon cancer[16, 17]. Those abnormalities were present in most $PTPN2^{flox/flox}$xVilcre animals but only in one $PTPN2^{flox/flox}$ mouse. Therefore, we considered the possibility that PTPN2 deficiency in IECs could mediate increased susceptibility to the development of colon cancer. This appeared plausible since EGFR is a known target of PTPN2. However, our DSS/AOM colitis experiment, a common model to study colon carcinogenesis, did not support this assumption. The combination of DSS treatment and AOM injection led to the development of aberrant crypt foci in nearly all animals irrespective of genotype. Our experiments may indicate that the loss of PTPN2 in epithelial cells in $PTPN2^{flox/flox}$xVilcre mice increases the susceptibility to aberrant crypts and dysplasia even under simple inflammatory conditions. These data point to a role of PTPN2 in IEC during colitis induction apart from simple inflammation. It may be more associated with epithelial repair. Further studies with different model systems will have to elucidate this IEC specific function.

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FIGURES

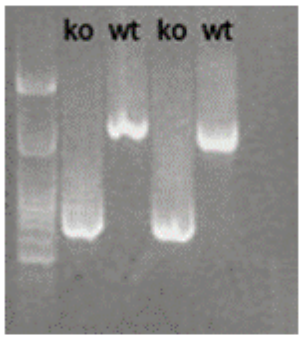


FIGURE 1: Conversion of the PTPN2 allele in IECs of PTPN2$^{flox/flox}$xVilcre mice. IECs were isolated from colon specimens of PTPN2$^{flox/flox}$xVilCre (ko) and PTPN2$^{flox/flox}$ (wt) mice. DNA was isolated from these IECs and used for genotyping PCR. Amplified PCR products were run in a 3 % agarose gel and 100 bp DNA ladder was used to estimate amplicon lengths.

Figure 2

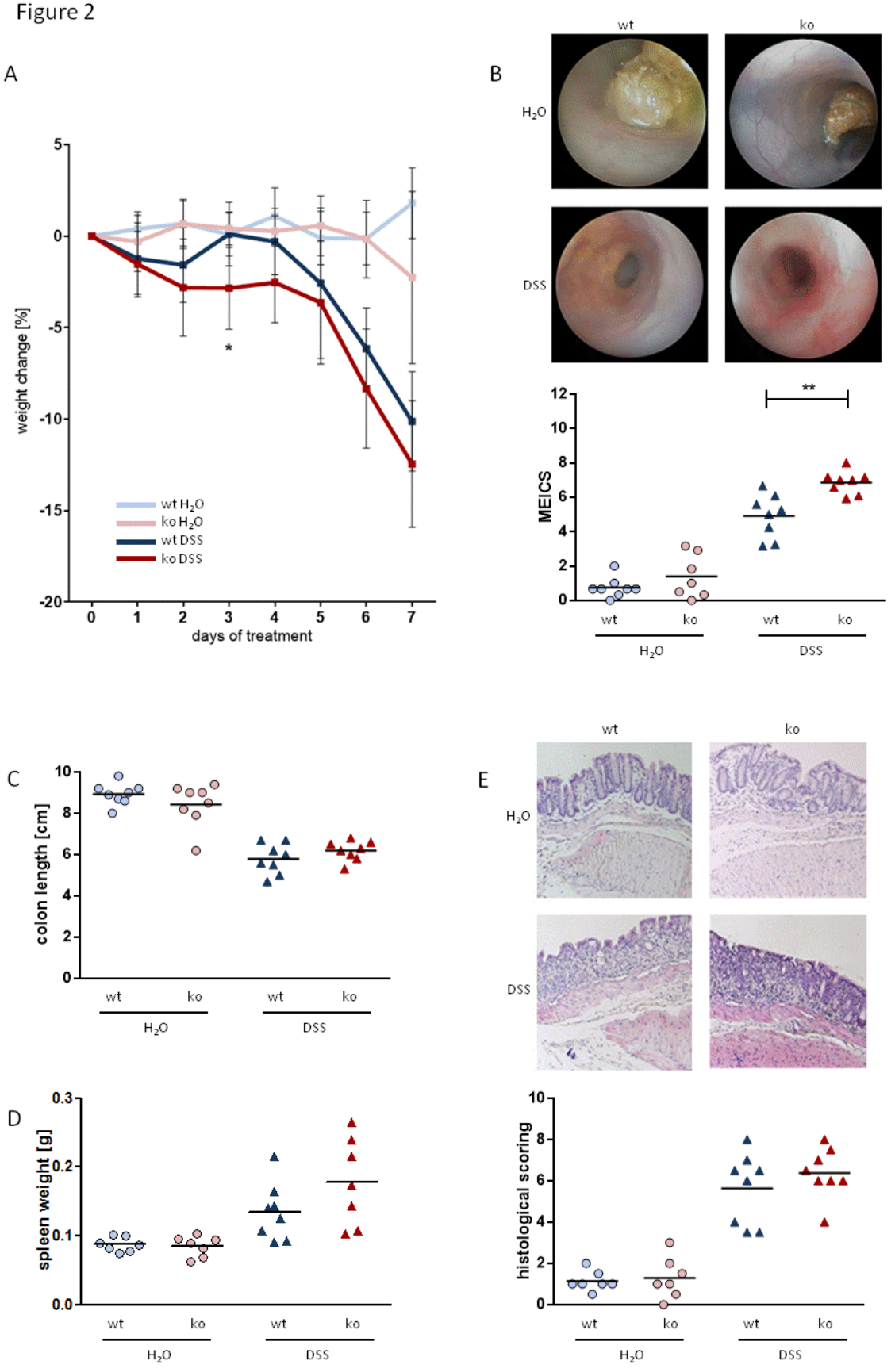

A
weight change [%]
days of treatment
wt H2O
ko H2O
wt DSS
ko DSS
*
B
wt
ko
H2O
DSS
MEICS
**
C
colon length [cm]
D
spleen weight [g]
E
histological scoring

FIGURE 2: PTPN2$^{flox/flox}$xVilcre mice tended to exhibit more severe inflammation in acute colitis using 2.5 % DSS. Female PTPN2$^{flox/flox}$xVilCre (ko) and their PTPN2$^{flox/flox}$ (wt) littermates received 2.5 % DSS for 7 days (n=8) and body weight was measured daily. (**A**) Percental weight changes indicate early onset of weight loss in PTPN2xVilCre mice, data are presented as mean ± SD. At day 7 colonoscopy was done and murine endoscopic index of colitis severity (MEICS) was used to determine inflammation severity. (**B**) Reperesentative colonoscopy pictures and MEICS show more severe DSS induced inflammation in PTPN2xVilCre mice. Mice were sacrificed, (**C**) colon length and (**D**) spleen weight was measured but both parameters were not influenced by genotype. Distal colon slides were used for H&E staining and scored for epithelial damage and infiltration. (**E**) Representative pictures and graph presenting histological score exhibit no difference between genotypes. Graphs B-E show values for individual mice, horizontal bars indicate mean, Mann Whitney U test was used for statistical analysis, * $p < 0.05$, ** $p < 0.001$ vs DSS treated wt group.

Figure 3

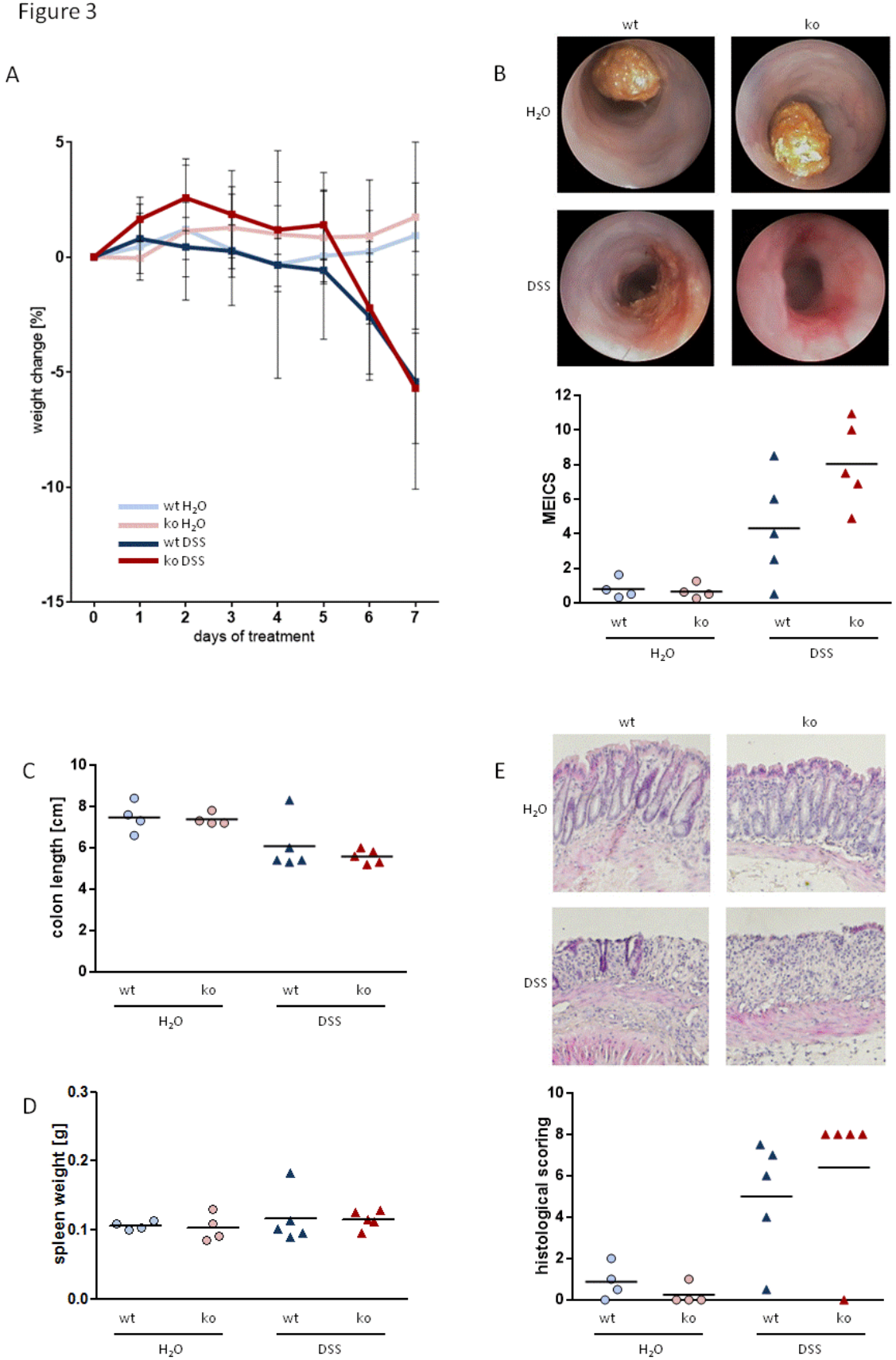
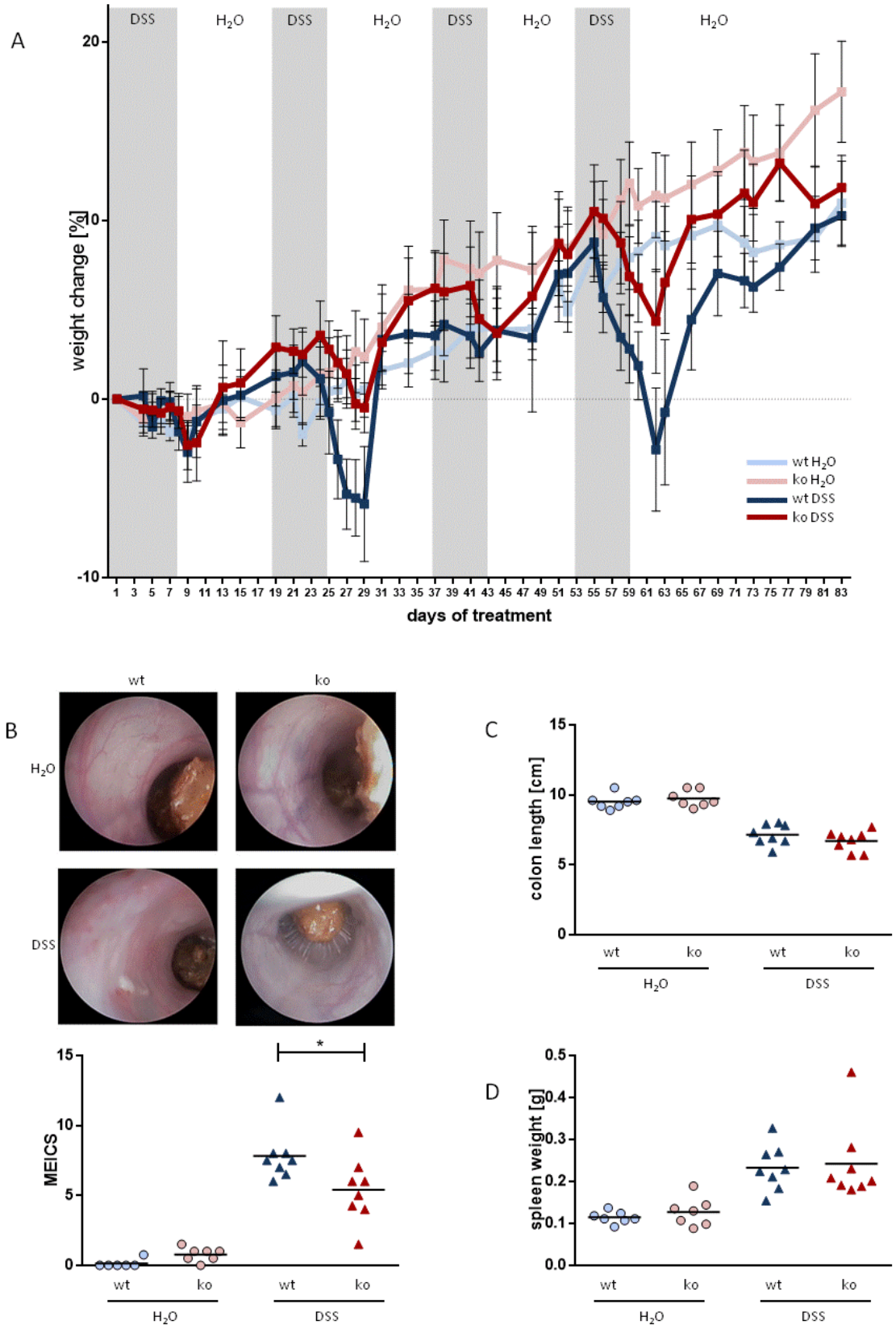

A
weight change [%]
days of treatment
wt H2O
ko H2O
wt DSS
ko DSS
B
wt
ko
H2O
DSS
MEICS
C
colon length [cm]
D
spleen weight [g]
E
histological scoring

FIGURE 3: No differences in inflammation severity in acute colitis using 2 % DSS. Female PTPN2$^{flox/flox}$xVilCre (ko) and their PTPN2$^{flox/flox}$ (wt) littermates received 2.5 % DSS for 7 days (n=4/5). Body weight was measured daily and (**A**) percental weight changes are presented as mean ± SD. At day 7 colonoscopy was done and murine endoscopic index of colitis severity (MEICS) was used to determine inflammation severity. (**B**) Reperesentative colonoscopy pictures and MEICS show no influence of PTPN2 deficiency. Mice were sacrificed, (**C**) colon length and (**D**) spleen weight was measured but both parameters were not altered by genotype. Distal colon slides were used for H&E staining and scored for epithelial damage and infiltration. (**E**) Representative pictures and graph presenting histological score exhibit no difference between genotypes. Graphs B-E show values for individual mice, horizontal bars indicate mean, Mann Whitney U test was used for statistical analysis.

Figure 4


A
DSS
H2O
DSS
H2O
DSS
H2O
DSS
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20
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0
-10
weight change [%]
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39 41 43 45 47 49 51 53 55 57 59 61 63 65 67 69 71 73 75 77 79 81 83
days of treatment
wt H2O
ko H2O
wt DSS
ko DSS
B
wt
ko
H2O
DSS
MEICS
15
10
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0
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wt
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H2O
DSS
C
colon length [cm]
15
10
5
0
wt
ko
wt
ko
H2O
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spleen weight [g]
0.5
0.4
0.3
0.2
0.1
0.0
wt
ko
wt
ko
H2O
DSS

FIGURE 4: PTPN2$^{flox/flox}$xVilcre mice tended to exhibit less severe inflammation in chronic DSS colitis. Female PTPN2$^{flox/flox}$xVilCre (ko) and their PTPN2$^{flox/flox}$ (wt) littermates received 4 cycles of DSS treatment and were allowed to recover afterwards for 3.5 weeks (n=7/8). Body weight was measured regularly and (**A**) percental weight changes are presented as mean ± SD. Colonoscopy was done at the end of the experiment and murine endoscopic index of colitis severity (MEICS) was used to determine inflammation severity. (**B**) Reperesentative colonoscopy pictures and MEICS show less severe inflammation in mice with PTPN2 deficiency in IECs. Mice were sacrificed, (**C**) colon length and (**D**) spleen weight was measured but both parameters were not altered by genotype. Graphs B-D show values for individual mice, horizontal bars indicate mean, Mann Whitney U test was used for statistical analysis, * $p < 0.05$.

Figure 5

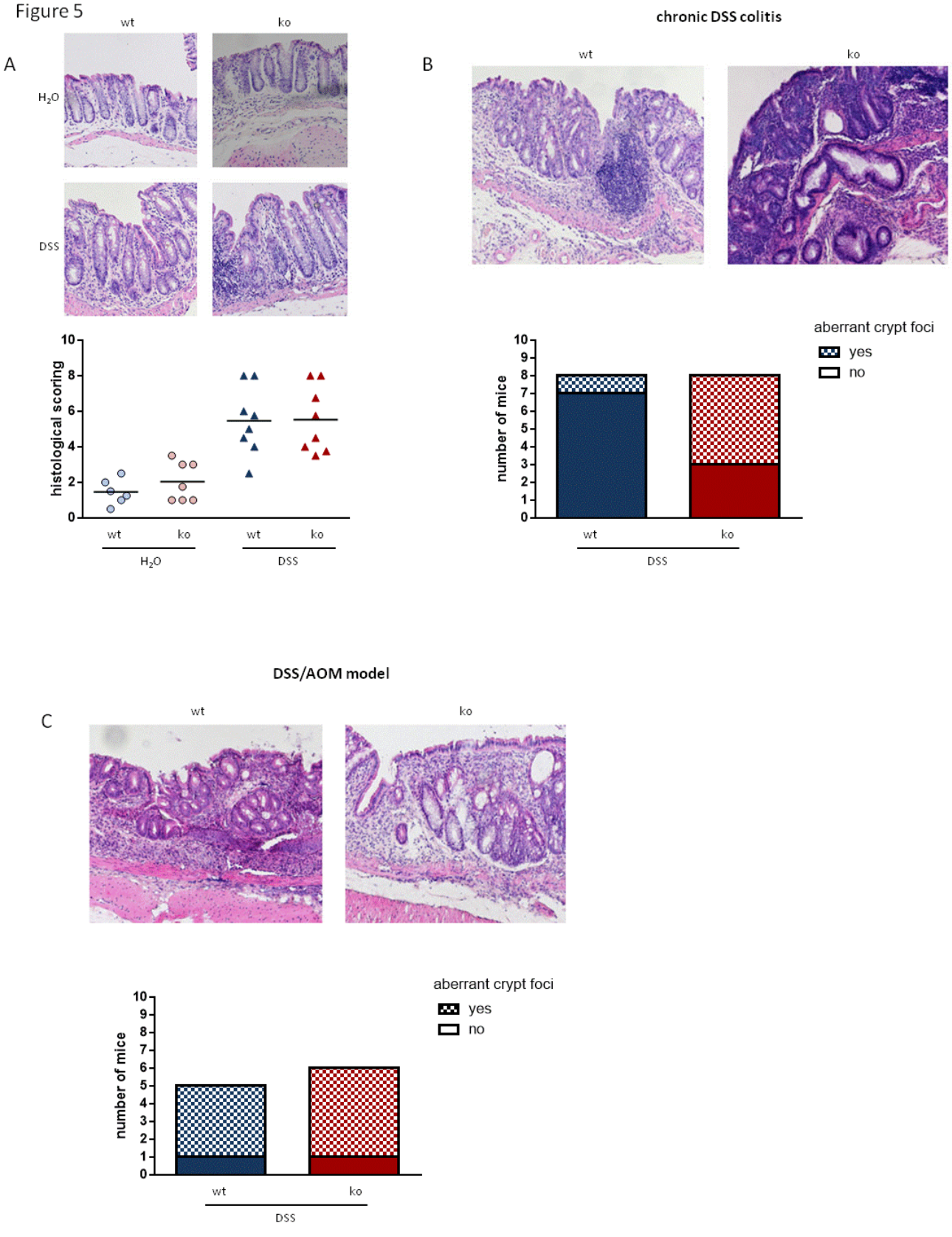
A
wt
ko
H2O
DSS
histological scoring
wt
ko
wt
ko
H2O
DSS
B
chronic DSS colitis
wt
ko
number of mice
aberrant crypt foci
yes
no
wt
ko
DSS
C
DSS/AOM model
wt
ko
number of mice
aberrant crypt foci
yes
no
wt
ko
DSS

FIGURE 5: PTPN2$^{flox/flox}$xVilcre mice developed more frequently aberrant crypt foci in chronic DSS colitis but not in a DSS/AOM model for colon cancerogenesis. To induce chronic colitis PTPN2$^{flox/flox}$xVilCre (ko) and their PTPN2$^{flox/flox}$ (wt) littermates received 4 cycles of DSS treatment and were allowed to recover afterwards for 3.5 weeks (n=7/8). At the end of the experiment mice were sacrificed and distal colon slides were used for H&E staining and scored for epithelial damage and infiltration. (**A**) Representative pictures and graph presenting histological score exhibit no difference between genotypes. Graph shows values for individual mice, horizontal bars indicate mean. (**B**) Aberrant crypt foci were detected more frequently in H&E stained slides of distal colon in PTPN2xVilCre mice. Colon carcinogenesis was induced by 4 cycles of DSS treatment combined with repeated AOM injections. (**C**) Aberrant crypt foci were found in nearly all DSS/AOM treated animal irrespective of genotype.

8 DISCUSSION

8.1 The cell type specific role of PTPN2 in ER stress signalling

In this work we showed that PTPN2 interferes with ER stress signalling in IECs and monocytes. Treatment with the ER stress inducer tuncamycin resulted in reduced PTPN2 protein levels in THP-1 and only slightly lowered expression in HT-29 cells. Bettaieb and colleagues used palmitate to create ER stress in pancreatic MIN6 cells and they observed impaired PTPN2 expression after 48h[63]. Knockdown of PTPN2 had beneficial effects in MIN6 cells, the magnitude of tunicamycin or palmitate stimulated ER stress was considerably alleviated and fewer cells underwent ER stress mediated apoptosis.[63] We observed the same advantageous impact of PTPN2 deficiency in HT-29 cells. In contrary, THP-1 cells suffered from increased ER stress susceptibility that was accompanied by higher apoptosis rate upon PTPN2 knockdown. It should be mentioned that we used HT-29 cells carrying the IBD associated PTPN2 variant. Therefore, we cannot be sure whether the observed effects were specific for intestinal epithelial cells or were mediated by the mutated PTPN2 gene. Nevertheless, we clearly demonstrated that under ER stress conditions the role of PTPN2 varies between cell types.

8.2 PTPN2 deficiency in IECs did not influence DSS colitis

In acute and chronic DSS colitis experiments, mice with IEC specific knockout of PTPN2 did not clearly differ from wild type mice. Even though colonoscopy indicated exaggerated inflammation in PTPN2xVilCre mice after acute colitis and alleviated inflammation after chronic colitis, histological analysis of colon slides did not resemble those differences. The histological scoring for epithelial damage and infiltration is regarded as the gold standard in evaluation of colitis severity, thereby negating the differences found during endoscopic examination. Hence, in IECs PTPN2 does not play an important role in inflammatory processes, at least not in the context of DSS induced colitis. Since global loss of PTPN2 is lethal and PTPN2 knockout mice die few weeks after birth on systemic inflammation[50], we expected our PTPN2xVilCre animals to be more susceptible to DSS. This theory was supported by cell culture experiments that highlight the importance of PTPN2 in autophagy, barrier function and cytokine secretion in IECs[54, 57, 59]. Obviously, PTPN2 deficiency in IECs can be compensated by the organism *in vivo*. Therefore, the severe phenotype of mice with total loss of PTPN2 seems to be primary mediated by immune cells. Other mouse studies were able to show that PTPN2 is important for $CD4^+$ cell development[89] and for TCR signalling and lymphopenia induced proliferation in $CD8^+$ cells[49, 60]. Apparently, lack of PTPN2 in the T cell compartment cannot be counterbalanced *in vivo*.

8.3 PTPN2 deficiency in IECS did not influence DSS/AOM colitis

During histological analysis after chronic colitis we observed the presence of aberrant crypt foci more frequently in DSS treated PTPN2xVilCre mice. This kind of epithelial transformation is usually observed after exposition to carcinogens and could develop into colon cancer[87, 88]. This led us to the assumption tissue specific knockout of PTPN2 in IECs could mediate increased susceptibility to colorectal cancer. With EGFR among the known PTPN2 substrates this would perfectly make sense. An experiment using the AOM/DSS model disproved our theory. The combined AOM and DSS treatment induced the formation of aberrant crypt foci in nearly all animals irrespective of genotype.

8.4 Concluding remarks and outlook

In our cell culture study PTPN2 knockdown was beneficial for HT-29 cell under ER stress conditions. Further experiments with another IEC cell line could clarify whether this observed effect is IEC specific or not. We used HT-29 cells that carry the IBD associated SNP and therefore we cannot exclude the possibility that our experiments were influenced by this mutation. We did not expect the observed alleviation of ER stress susceptibility upon PTPN2 knockdown because this would be contrary to the task of a risk gene. Even though lack of PTPN2 in IECs is reportedly disadvantageous for autophagy[57], barrier function[54] and cytokine secretion[59], it is still possible that it may be beneficial in other pathways.

In the context of DSS colitis the tissue specific loss of PTPN2 in IECs did not influence inflammation. It would be interesting to use our PTPN2xVilCre mouse in another colitis model, maybe breeding into IL-10$^{-/-}$ background could reveal a phenotype.

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11 CURRICULUM VITAE

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Research experience

2011	**PhD thesis "The role of PTPN2 in inflammatory bowel diseases"** Methods: cell culture (THP-1, HT-29, Caco-2), mouse models (DSS colitis, DSS/AOM model), Western blot, ELISA, quantitative real-time PCR, immunohistochemistry, immunofluorescence Software: iRats, Photoshop, GraphPad Prism, optiquant Dept. of Gastroenterology and Hepatology, University Hospital Zurich Supervisor: PD Dr. med. Michael Scharl
2009 - 2010	**Diploma thesis "Expression patterns of voltage gated sodium channels in the olfactory system of mouse"** Methods and software: transcardial perfusion, cryoembedding and cutting, immunofluorescence, photoshop Dept. of Physiology, University Hospital Homburg Supervisor: Dr. Martina Pyrski
2008 July	**Student assistant job: general laboratory work** Methods: genotyping PCRs, bisulphite sequencing Dept. of Genetics/Epigenetics, Saarbruecken University Supervisor: Dr. Sascha Tierling

Teaching experience

	Teaching assistances in practical Physiology courses at Zurich University **within the framework of my doctoral studies**
2015	"Intracellular Structures"
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2013	"Heart ECG"
2008	**Student assistant job**, teaching assistance in the practical course on "Developmental Biology II" (Prof. Dr. Waldorf, Saarland University Saarbruecken)
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General Education

2011 - 2015	**PhD** student **at University Hospital Zurich** Dept. of Gastroenterology and Hepatology
2004 - 2010	Molecular **and Human Biology (Diploma)** Saarland University Saarbruecken final grade: 2,0 (in a system where 1 is the best and 4 is the lowest positive score)
2001 - 2004	**Abitur (university-entrance diploma)** "Abendgymnasium Saarbruecken" (evening school)
1999 - 2002	**Training as „Kauffrau für Bürokommunikation"** (Management Assistant in Office Communication) Esser & Co. Saar GmbH in Rilchingen-Hanweiler
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Languages

German:	native speaker
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French:	basic

Conference Presentations

Oral Presentation

May 2014	Digestive **Disease Week, Chicago, Illinois** "Mice with loss of PTPN2 in the colon epithelium show increased mucosal inflammation" Travel Grant Hartmann Mueller Stiftung

Poster Presentation

Feb 2013	Interdisciplinary **Symposium Inflammation at Interfaces, Hamburg** "The IBD susceptibility gene PTPN2 is involved in ER stress signaling in HT-29 and THP-1 cells" Travel Grant Abbott
May 2013	**Digestive Disease Week, Orlando, Florida** "The IBD susceptibility gene PTPN2 is involved in ER stress signaling in human IECs and monocytes" Travel Grant Hartmann Mueller Stiftung
April 2013	**Clinical** Research **Day, Zurich** "The IBD susceptibility gene PTPN2 is involved in ER stress signaling in HT-29 and THP-1 cells"
March 2014	**World** Immune **Regulation Meeting, Davos** "Mice with a loss of PTPN2 in the colon epithelium show increased mucosal inflammation in acute DSS colitis"
March 2015	**World** Immune **Regulation Meeting, Davos** "Mice with loss of PTPN2 in IECs show epithelial transformations after chronic DSS colitis"
May 2015	**Digestive** Disease **Week, Washington, DC** "Mice with loss of PTPN2 in IECs show epithelial transformations after chronic DSS colitis"

Publications

Kasper SH, Spalinger MR, Raselli T, Scharl M; "A Cell Type-Specific Role of Protein Tyrosine Phosphatase Non-Receptor Type 2 in Regulating ER Stress Signaling" *Digestion,* 2015 Mar 25;91(3):248-256

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